

Evaluation of phytase enzymes on performance, bone mineralisation, carcass characteristics and small intestinal morphology of broilers fed maize soya bean diets

by

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Declaration

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Summary

The use of phytase enzymes to liberate phosphorus and other phytate bound nutrients in monogastric animal diets are becoming common practice and several commercial phytase enzymes are available on the market. Phytase manufacturers supply nutritionists with matrix values for the specific phytase, enabling nutritionists to effectively decrease the dietary concentrations of phosphorus and nutrients during diet formulation. A 32 day experiment was conducted with 5120 broiler chicks fed diets supplemented with different commercial phytase enzymes (1000 FYT or 1500 FYT HiPhos/kg diet, 1500 FYT Ronozyme/kg diet, 500 FTU Natuphos/kg diet or 500 FTU Phyzyme/kg diet) at levels recommended by the manufacturers and with similar phosphorus equivalence. The nutrient content of the diets supplemented with 500 FTU Natuphos, 500 FTU Phyzyme 1500 Ronozyme and 1000 FYT HiPhos were reduced according to the matrix values of 1000 FYT/kg HiPhos, whilst the diet supplemented with 1500 FYT HiPhos /kg diet was reduced according to the matrix values 1500 FYT HiPhos. The objectives of this study were threefold: (i) to confirm the matrix value for a newly developed phytase (HiPhos, DSM Nutritional Products, Basel, Switzerland), at two different inclusion levels, using weight gain and bone parameters of broilers as response criteria; (ii) to compare production and bone parameters of broilers reared on three different commercial phytases to broilers reared on HiPhos (iii) to investigate the effect that supplementation of these four phytases has on water intake, carcass characteristics, organ weights and gastrointestinal tract morphology of broilers. The matrix values for 1500 FYT HiPhos were confirmed by using live weight gain as response criteria, but results for bone parameters were insufficient in confirming the matrix values. The matrix values for 1000 FYT HiPhos were confirmed by the results for tibia weight and tibia strength, but results for weight gain were insufficient to confirm the values. The matrix values for 1000 FYT HiPhos and 1500 FYT HiPhos could not be confirmed nor disproved, nevertheless results from the current trial proved diets supplemented with HiPhos to be more economically viable when compared to the standard commercial broiler diet. Total feed and water intake were not influenced by phytase supplementation. Production parameters (live weight, feed intake, feed conversion ratio, European production efficiency factor and average daily gain) and bone parameters (tibia strength, fat free tibia weight, fat free tibia ash and mineral content) did not differ between phytase treatments and therefore all the commercial phytases were equally effective to the HiPhos phytase. Furthermore, results indicate that the investigated phytases had no effect on internal organ weight or gastrointestinal tract morphology in broilers. Overall the results obtained from the study indicate that the use of phytase as feed additive has no negative effects on growth performance, carcass characteristics or bone parameters. No major differences for the production and bone parameters were observed between broilers supplemented with different phytases. Therefore the costs of these phytases can be the determining factor when nutritionists decide which commercial phytase to use.

Opsomming

Die gebruik van fitase ensieme in die diëte van enkelmaagdiere om fitaat-gebonde fosfor en voedingstowwe vry te stel, word al hoe meer algemeen in die bedryf en verskeie kommersiële fitase ensieme is in die mark beskikbaar. Die ensiemvervaardigers verskaf die fitases se matryswaardes aan voedingskundiges wat hul in staat stel om die fosfor- en nutrientvlakke in die dieet effektief te verminder. 'n Studie met 5120 braaikuikens was vir 32 dae uitgevoer. Die braaikuiken diëte was met verskillende kommersiële fitase ensieme (1000 FYT & 1500 FYT HiPhos/kg dieet, 1500 FYT Ronozyme/kg dieet, 500 FTU Natuphos/kg dieet of 500 FTU Phyzyme/kg dieet) aangevul. Die nutrientvlakke van die diëte wat met fitase aangevul was, was verminder volgens die matryswaardes van 1000 FYT of 1500 FYT HiPhos fitase. Die doelstellings van hierdie studie was drievoudig: (i) om die matryswaardes van 'n nuwe fitase (HiPhos, DSM Nutritional Products, Basel, Switzerland) by twee verskillende insluitingsvlakke te bevestig deur massa toename en been parameters as reaksie maatstawwe te gebruik (ii) om produksie- en been parameters van braaikuikens, wat een van drie kommersiële fitase ensieme as voerbymiddel ontvang het, met dié van braaikuikens wat die nuwe ensiem gevoer was te vergelyk (iii) om die effek wat fitase op water inname, karkaseienskappe, orgaan massas en spysverteringskanaal morfologie het te bestudeer. Die matryswaardes vir 1500 FYT HiPhos was bevestig deur lewendige massa toename as respons kriteria te gebruik, maar resultate vir die been parameters was onvoldoende om die matryswaardes te bevestig. Die matryswaardes vir 1000 FYT HiPhos was slegs bevestig deur die resultate vir die breeksterktes van die tibias, maar resultate vir massa toename was onvoldoende om die matryswaardes te bevestig. Dus kon die matryswaardes vir die HiPhos fitase nie bevestig of verkeerd bewys word nie. Desondanks het die resultate in die huidige proef bewys dat diëte wat met HiPhos aangevul was meer ekonomies as die kommersiële braaikuiken dieet is. Totale voer- en water-inname was nie deur die aanvulling van fitase beïnvloed nie. Produksie parameters (lewendige massa, voeromset, die Europese produksie doeltreffendheids faktor, gemiddelde daaglikse toename) en been parameters (tibia breeksterkte, vet vrye tibia massa, vet vrye tibia as en mineraal-inhoud) het nie verskil tussen die fitase behandelings nie en dus was al die kommersiële fitases ewe effektief. Vanuit die studie is getoon dat die gebruik van fitase as 'n voerbymiddels geen negatiewe effek op groei, karkas eienskappe of been parameters het nie en dat fitase ook nie die orgaan gewigte of die spysverteringskanaal morfologie van braaikuikens beïnvloed nie. Geen groot verskille in produksie- en been parameters was waargeneem tussen hoenders wat verskillende fitases as voerbymiddel ontvang het nie, daarom kan die koste van die ensiem die bepalende faktor wees as voedingskundiges die keuse maak tussen hierdie kommersiële fitases.

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Notes

The language and style used in this thesis are in accordance with the requirements of the *South African Journal of Animal Science*. This thesis represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters is therefore unavoidable.

Abbreviations

ADG	Average daily gain
µm	Micrometer
AME	Apparent metabolisable energy
ANOVA	Analysis of variance
aP	Available phosphorus (The bioavailable phosphorus determined using a slope ratio assay and expressed relative to monocalcium phosphate)
Cl ⁻	Chloride
Ca	Calcium
Co	Cobalt
CP	Crude protein
Cu	Copper
DEB	Dietary electrolyte balance
dP	Digestible phosphorus
EPEF	European production efficiency factor
FCR	Feed conversion ratio
Fe	Iron
FTU	Phytase units (standard unit)
FYT	Phytase units (phytase units for phytases from DSM)
g	Gram
IP1	Myo-inositol monophosphate
IP2	Myo-inositol bisphosphate
IP3	Myo-inositol trisphosphate
IP4	Myo-inositol tetrakisphosphate
IP5	Myo-inositol pentakisphosphate
IP6	Myo-inositol hexakisphosphate (phytate)
K	Potassium
kg	Kilogram
L	Litres
meq/kg	Milliequivalents of solute per kilogram
Mg	Magnesium
Mn	Manganese
N	Newton
N/g	Newton per gram
Na	Sodium
Ni	Nickel
npP	Non phytate phosphorus (Analysed total phosphorus less the phosphorus from phytate)
NRC	National Research Council
P	Phosphorus
Phytate-P	Phytate bound phosphorus
pH _i	pH 15 minutes <i>post mortem</i> (initial pH)
pH _u	pH 24 hours <i>post mortem</i> (ultimate pH)
PO ₄ ³⁻	Orthophosphate
SAPA	South African Poultry Association
tP	Total phosphorus
ZAR	South African Rand
Zn	Zinc

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Chapter 1

Introduction

The poultry industry in South Africa accounted for 65.5% of the locally produced animal protein in 2012, making it the largest agricultural sector in the country (SAPA, 2013). According to figures from the South African Poultry Association (SAPA), the poultry industry supplied 1884690 tons of poultry meat in 2012 (SAPA, 2013). Regardless of these figures, the broiler industry experienced a crisis during the past two years. Margins were significantly reduced due to a large increase in imported products together with higher feed costs ($\pm 30.5\%$) due to the unexpected rise in grain prices. Eventually the rise in production costs will be transmitted to the consumer. At the moment poultry meat is the most affordable source of animal protein. However, the rising price of poultry meat makes it impossible for the poor to afford a good quality protein source and as a result the nutritional status of vulnerable groups, such as children and immunity impaired individuals, may be negatively affected. Therefore strategies to decrease the cost of production are extremely important. Reducing feed costs while retaining the quality of the feed is one possibility to explore.

Phosphorus (P) is an essential element needed for proper development of the chicken because of its importance as a constituent of the skeleton and its key role in several metabolic processes (Suttle, 2010). It is therefore important that the P level in the animal's diet meets its daily requirements. Feed phosphates are expensive feed ingredients in poultry diets, but have to be incorporated into the diets due to the low bioavailability of P in cereals (maize) and oilseeds (soya beans), which forms part of the primary ingredients in broiler diets in South Africa. Generally cereals and oilseeds are rich in P, but the majority of the P is in the form of phytate (Ravindran *et al.*, 1994). The phytate bound P is essentially unavailable for digestion and absorption by monogastric animals and as a result ends up being excreted. Excess P levels in poultry manure can leach into lakes and streams and contribute to environmental pollution (Nahm, 2007). In addition, phytate has the ability to form complexes with other minerals (Davies & Olpin, 1979; Cheryan & Rackis, 1980; Lonnerdal *et al.*, 1989; Brink *et al.*, 1991), protein (Hídvégi & Lásztity, 2002) and starch (Yoon *et al.*, 1983) in the diet, rendering these nutrients unavailable for absorption.

Phytase is the only enzyme capable of hydrolysing phytate; thereby releasing the phytate bound P and nutrients. In 1991, the first microbial phytase was commercially available as a feed additive. Since 1991, a number of phytase enzymes have been developed from different strains of micro-organisms. Consequently, broiler diets supplemented with phytase can be formulated with lower levels of feed phosphates, amino acids, crude protein (CP), metabolisable energy (ME) and minerals, resulting in lower feed costs. Nutritionists rely on matrix values from the manufacturer to determine how much P and nutrients can be reduced in the diet. These matrix values are derived from numerous feeding and digestibility trials.

The purpose of this study was threefold:

The main objective of the study was to confirm the matrix values of a newly developed phytase (HiPhos) when supplemented to broilers fed a maize soya bean diet. Growth and bone mineralisation are normally influenced by dietary P levels and are sensitive indicators of mineral adequacy in the diet. Therefore production parameters and bone mineralisation was used as the response criteria in confirming the matrix values.

A secondary objective was to compare three commercial phytases with the newly developed phytase to determine if these phytases have the ability to achieve the matrix values of the new phytase. Production parameters and bone mineralisation were used as response criteria.

The last objective was to investigate the effect of these commercial phytases and the newly developed phytase on water intake, carcass characteristics, meat quality characteristics, organ weights and lastly their effect on the gastrointestinal tract of broilers.

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Chapter 2

Literature Review

2.1 Introduction

Phosphorus (P) is an essential element for all animals and has more known functions in the body than any other mineral (McDonald *et al.*, 2002). Together with Calcium (Ca), it forms the structural component of the skeleton (Pond *et al.*, 2005). Phosphorus, which is a component of the cell wall and a constituent of several enzyme systems (McDonald *et al.*, 2002), plays a vital role in energy metabolism and has an influence on voluntary feed intake (Bar & Hurwitz, 1984). Therefore P is an essential mineral needed for the proper development of animals.

In South Africa poultry diets are mainly plant based, consisting of maize and soya bean meal. Plants are a major source of dietary P, but approximately 60-75% of the total P (tP) in these common feed ingredients is bound as phytate (Selle & Ravindran, 2007). Phytate bound P (phytate-P) is largely unavailable to monogastric animals and therefore P should be added to the diet in the form of feed phosphates to compensate for the lack of available dietary phosphorus. Additionally, phytate has the ability to bind other minerals (Zn^{2+} , Cu^{2+} , Ni^{2+} , Co^{2+} , Mn^{2+} , Ca^{2+} , Fe^{2+} , K^{2+} , Mg^{2+}) and nutrients (protein, amino acids, starch), rendering them unavailable for absorption (Cheryan & Rackis, 1980). Unabsorbed P and nutrients are excreted in the faeces (Nahm, 2007) and therefore have to be supplemented in greater amounts, subsequently increasing the cost of production. Furthermore, P levels exceeding crop requirements can result in P leaching into streams and lakes. The inorganic nutrients promote algae growth (eutrophication), which poses a threat to fresh water and marine ecosystems (Nahm, 2007). It is therefore important to increase the bioavailability of P in the gastrointestinal tract of monogastric animals. An approach to achieve this objective is the supplementation of animal diets with exogenous phytase.

Phytase is a naturally occurring enzyme and is the only enzyme known to release phosphates from phytate (Greiner & Konietzny, 2011), rendering it available for absorption. Due to increased cost of feed phosphates (Ahmad *et al.*, 2000) and legislations designed to limit P pollution (for example as found in the Netherlands), there was pressure in developing and releasing a commercially available phytase enzyme as early as 1991 (Selle & Ravindran, 2007). Since then a number of commercial phytase enzymes have been developed. Over the past two decades, the efficiency of commercial phytases has been widely investigated. Commercial phytases differ in origin, the temperature at which optimum phytase activity occurs (temperature optimum), temperature stability, pH profile and proteolytic resistance (Greiner & Konietzny, 2011). As a result there are many environmental factors affecting phytase efficiency and thus the *in vivo* dephosphorylation of phytate.

2.2 Phytate

Phytate, the mixed cation salt of phytic acid (Myo-inositol-1,2,3,4,5,6-hexakis dihydrogen phosphate), is a naturally occurring compound present in feedstuffs of plant origin (Reddy *et al.*, 1982). Phytate was identified by Hartig (1855) who isolated small, unknown particles from a variety of plant seeds (Reddy *et al.*, 1982). Phytate serves as the primary storage form of P and inositol in seeds (Hídvégi & Lásztity, 2002). It is also involved in controlling homeostasis of P levels in seeds (Lott *et al.*, 2000) and plays an important role in plant growth and seed germination (Aureli *et al.*, 2011).

In some plants, phytic acids binds potassium (K^{2+}), magnesium (Mg^{2+}) and to a lesser extend calcium (Ca^{2+}) to form phytin (Maenz, 2001). Phytin is stored in vacuoles known as protein bodies. It is distributed in dense aggregates called globoids or can be distributed throughout the proteinaceous matrix (Maenz, 2001). Phytate accumulates in the aleurone layer in monocotyledonous seeds (wheat, rice, barley) and in the germ of corn (Hídvégi & Lásztity, 2002). The amount of phytate in plant sources is influenced by cultivar and climatic conditions. Phytate is located in the outer parts of the kernel and therefore different milling methods can also influence the phytate content of the end products (Hídvégi & Lásztity, 2002). Phytate levels can be measured through the use of high performance liquid chromatography (HPLC) and the amount of phytate bound P can be calculated as 28.2% of the total phytate concentration (Sauvant *et al.*, 2004).

2.2.1 Structure of phytate

Phytic acid is a charged molecule and consists out of a myo-inositol ring (a six carbon molecule) and six phosphate groups extending from the structure (Johnson & Tate, 1969). The molecule has 12 proton dissociation sites with a high chelation capacity for multivalent cations (Cheryan & Rackis, 1980) and positively charged nutrients (Selle & Ravindran, 2007). The structure of phytate and possible bonds it may form is illustrated in Figure 2.1. At neutral pH, phytic acid can have one or two negatively charged oxygen atoms in the phosphate groups. Therefore there is likely to be a strong chelation interaction between cations and two phosphate groups and also a weak chelation interaction between cations and a single phosphate group (Singh, 2008).

2.2.2 Effects of phytate on mineral utilization

About two thirds of the P content in plants is in the form of phytate-P (NRC, 1994). It is generally accepted that phytate-P is poorly digested by poultry. Different raw materials contain different levels of phytate-P and the part of the plant from which the feedstuff is derived from influences this amount. For instance, cereal by-products and oil seed meals contain larger amounts of phytate-P compared to legumes and grains (Singh, 2008). Selle & Ravindran (2007) reviewed multiple papers and summarized the proportions of phytate-P in poultry feed ingredients as shown in Table 2.1.

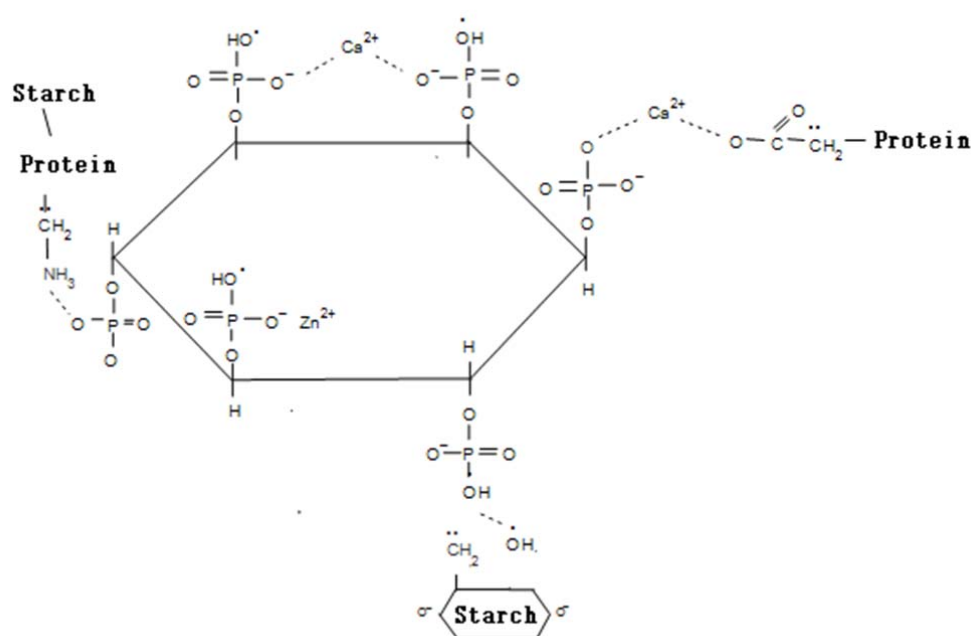


Figure 2.1 Phytate molecule and possible interactions with nutrients (modified after Thompson, 1986)

Table 2.1 Weighted mean (and range) of total P and phytate-P concentrations, and proportions of phytate-P of total P, in poultry feed ingredients (Selle & Ravindran, 2007)

Feed ingredient	Total P (g/kg)	Phytate-P (g/kg)	Proportion (%)
Cereals			
Barley	3.21 (2.73 - 3.70)	1.96 (1.86 - 2.20)	61.00 (59 - 68)
Maize	2.62 (2.30 - 2.90)	1.88 (1.70 - 2.20)	71.60 (66 - 85)
Sorghum	3.01 (2.60 - 3.09)	2.18 (1.70 - 2.46)	72.60 (65 - 83)
Wheat	3.07 (2.90 - 4.09)	2.19 (1.80 - 2.89)	71.60 (55 - 79)
Oilseed meals			
Canola meal	9.72 (8.79 - 11.50)	6.45 (4.00 - 7.78)	66.40 (36 - 76)
Cottonseed meal	10.02 (6.40 -11.36)	7.72 (4.90 - 9.11)	77.10 (70 - 80)
Soya bean meal	6.49 (5.70 - 6.94)	3.88 (3.54 - 4.53)	59.90 (53 - 68)
By-products			
Rice bran	17.82 (13.40 -27.19)	14.17 (7.90 - 24.20)	79.50 (42 - 90)
Wheat bran	10.96 (8.02 -13.71)	8.36 (7.00 - 9.60)	76.30 (50 - 87)

Poultry absorbs P in the inorganic form (phosphate, PO_4^{3-}) and therefore the ability to hydrolyse phytate in the gastrointestinal tract will affect their ability to utilize phytate-P (Singh, 2008). The nature of phytate hydrolysis is complex and this may be the reason for the wide variability in reports as to the efficacy of phytate digestion by poultry (Singh, 2008). It was first believed that poultry utilizes phytate bound-P very poorly (Nelson, 1967), but studies in later years demonstrated that poultry are capable of digesting more phytate in larger amounts than was believed in previous years. Mohammed *et al.* (1991) reported a phytate digestibility of 50% by means of endogenous phytase, whereas Ballam *et al.* (1984) reported phytate hydrolysis ranging from 3 to 42% depending on the source of fibre added to a maize and soya bean diet and the dietary calcium content. The effect of Ca on phytate hydrolysis will be discussed in section 2.4.1.1.

Phytic acid creates complexes with multivalent (divalent or trivalent) cations to form insoluble salts at neutral pH (Cheryan & Rackis, 1980) thereby potentially rendering these minerals unavailable for absorption (Singh, 2008). It is known that phytic acid decreases the bioavailability of Ca (Lonnerdal *et al.*, 1989), Mg (Brink *et al.*, 1991), Zn (Davies & Olpin, 1979; Lonnerdal *et al.*, 1989) and Fe (Brune *et al.*, 1992) which are all nutritionally important minerals. In descending order of stability, most stable complexes and insoluble salts are formed between phytic acid and Zn^{2+} followed by Cu^{2+} , Ni^{2+} , Co^{2+} , Mn^{2+} , Ca^{2+} and Fe^{2+} (Cheryan & Rackis, 1980).

High dietary levels of phytic acid increases the Ca requirements in monogastric animals (Singh, 2008). Theoretically, the phytic acid molecule has 12 proton dissociation sites (Cheryan & Rackis, 1980); hence, it has the potential to chelate six Ca atoms (Selle *et al.*, 2009). Even though the affinity of phytic acid is greater for other divalent cations, Ca levels in animal diets are the highest compared to the other divalent cations and therefore the formation of Ca-phytate complexes in the gastrointestinal tract are important to acknowledge (Selle *et al.*, 2009).

Complexes with decreased solubility are less readily degraded by means of phytase (Nolan *et al.*, 1987). Zinc forms an insoluble complex with phytate at pH 6, the pH of the intestine, and can become a limiting mineral in diets with high levels of phytate (Maddaiah *et al.*, 1964). Ca-phytate complexes precipitate at pH 5, but are still soluble at a pH below 4 (Wise & Gilbert, 1981). On average a Ca-phytate complex contains 4.93 Ca atoms per molecule phytate (Marini *et al.*, 1985). Poultry diets typically contain Ca and phytate levels of 10g/kg and theoretically one third of the Ca forms complexes with phytate (Selle *et al.*, 2009).

2.2.3 Binding property of phytate to protein

Rojas & Scott (1969) were the first to suggest that phytate might have a negative effect on protein utilisation. When the lumen pH of the gastrointestinal tract is below the isoelectric point of proteins, binary protein-phytate complexes can form between the negatively charged phosphate groups on phytate and positively charged terminal amino groups on proteins (Hídvégi & Lásztity, 2002). The pH in the proventriculus of chickens is low and a low pH is ideal for the formation of binary protein-phytate complexes. These complexes decrease the solubility of protein. Excess Ca can interact with protein-phytate complexes and decrease the solubility even further (Saio *et al.*, 1967). However,

supplementing broilers diets with exogenous phytase can hydrolyse phytate in the crop and partially prevent the formation of these complexes (Selle & Ravindran, 2007). The formation of protein-phytate complexes in feedstuffs is possible but unlikely because a low pH is required for the formation of this complex (Selle *et al.*, 2006).

Proteins and phytate have a net negative charge at a pH higher than five and therefore it is unusual to have interactions between these molecules at high pH levels. In the presence of multivalent cations (Ca^{2+} , Mg^{2+} , Zn^{2+}), bridges form between the negatively charged carboxyl groups on a protein and the negatively charged phosphate groups on phytate, allowing proteins and phytate to bind at neutral pH to form ternary protein-mineral-phytate complexes (Hídvégi & Lásztity, 2002; Champagne *et al.*, 1990). These insoluble complexes are refractory to pepsin activity (Vaintraub & Bulmaga, 1991; Knuckles *et al.*, 2006) and might be one of the reasons why phytate has a negative influence on the digestibility of protein (Selle *et al.*, 2000).

It is believed that phytate may have the ability to inhibit digestive enzymes (Maenz, 2001). Singh & Krikorian (1982) proposed that phytate alters the protein configuration of proteolytic enzymes and inhibits proteolysis. *In vitro* trials have been done to determine the effect of phytate on trypsin activity, but the results in literature are inconsistent (Singh & Krikorian, 1982; Deshpande & Damodaran, 1989; Vaintraub & Bulmaga, 1991; Caldwell, 1992). Furthermore, it is questionable if there is sufficient free phytate in the small intestine to interact and inhibit trypsin activity (Sajjadi & Carter, 2004). *In vivo* studies in fish however supports the theory that phytate decreases trypsin activity, but there is a lack of supportive *in vivo* data (Selle *et al.*, 2000). In addition to the binding properties of phytate to protein, it has been shown that the addition of 1 g phytic acid to broilers fed only on glucose can increase the excretion of endogenous nitrogen, amino acids, sialic acid, sulphur, sodium and iron. This phenomenon was due to increased mucin (rich in certain amino acids), pancreas and gallbladder excretions (Cowieson *et al.*, 2004).

2.2.4 Binding property of phytate to starch

The apparent metabolisable energy (AME) of a diet decreases as the dietary level of phytate increases (Ravindran *et al.*, 2006). The possibility exists that phytate can bind with starch through phosphate linkages, but there is little information on the interaction between these compounds (Yoon *et al.*, 1983). Phytate does however decrease starch digestibility and *in vivo* glucose levels (glycaemic index) in humans. There are three possible explanations to the phenomenon: (i) the direct binding of phytate to starch; (ii) the binding of phytate to protein closely associated with starch; (iii) the binding of phytate to amylase or to Ca (which catalyses amylase activity), however the reason is unclear (Yoon *et al.*, 1983; Thompson & Yoon, 1984).

2.3 Phytase

Phytase is a subgroup of phosphomonoesterases and initiates the stepwise dephosphorylation of phytate (Greiner & Konietzny, 2011). So far phytase is the only enzyme recognised to release

inorganic phosphate (PO_4) from phytate (Shaw *et al.*, 2010). Monogastric animals absorb P in the form of orthophosphate (PO_4^{3-}) (Greiner & Konietzny, 2011) and are reliant on phytase enzymes to utilize phytate as a source of P. Authors often assume animals not to possess the ability to synthesise phytase enzymes, however a specific phytase activity has been demonstrated in the brush border membrane in the small intestine of chicks (Maenz & Classen, 1998); nevertheless, phytate-P is still poorly digested by poultry (Ballam *et al.*, 1984). Therefore the development of exogenous phytases was a very important discovery in animal nutrition (Cromwell, 2009).

Multiple forms of phytases are synthesised by microorganisms. These enzymes may exhibit different stereospecificity for phytate dephosphorylation and may have different physiological functions. Extracellular phytases of yeast and molds are triggered by phosphate starvation. These enzymes hydrolyse organic phosphorylated compounds, for example phytate, to provide phosphate from extracellular sources to the cell. These enzymes are therefore non-specific phosphatases that also exhibit phytate degrading ability (Greiner & Konietzny, 2011).

2.3.1 Phytase dephosphorylation

The hydrolysis of phytate (IP6) by means of phytase takes place via a pathway of stepwise dephosphorylation resulting in myo-inositol pentakis- (IP5), tetrakis- (IP4), tris- (IP3), bis- (IP2), and monophosphates (IP1) (Wyss *et al.*, 1999 & Greiner *et al.*, 2000; Greiner *et al.*, 2001). Orthophosphate and partially phosphorylated myo-inositol phosphates are the products of hydrolysis (Konietzny & Greiner, 2002). The general enzymatic reaction is shown in Figure 2.2. Phytase enzymes release reaction intermediates, which are able to serve as a substrate for further hydrolysis. None of the histidine acid phytases synthesised by bacteria or fungi are able to dephosphorylate the complete myo-inositol ring. The phosphate residue in position C₂ is usually resistant to dephosphorylation and therefore only five of the six phosphate residues are normally released (Wyss *et al.*, 1999; Greiner *et al.*, 2000; Greiner *et al.*, 2001). After the first phosphate residue is removed, histidine acid phytases dephosphorylate the adjacent hydroxyl group (Greiner & Konietzny, 2011).

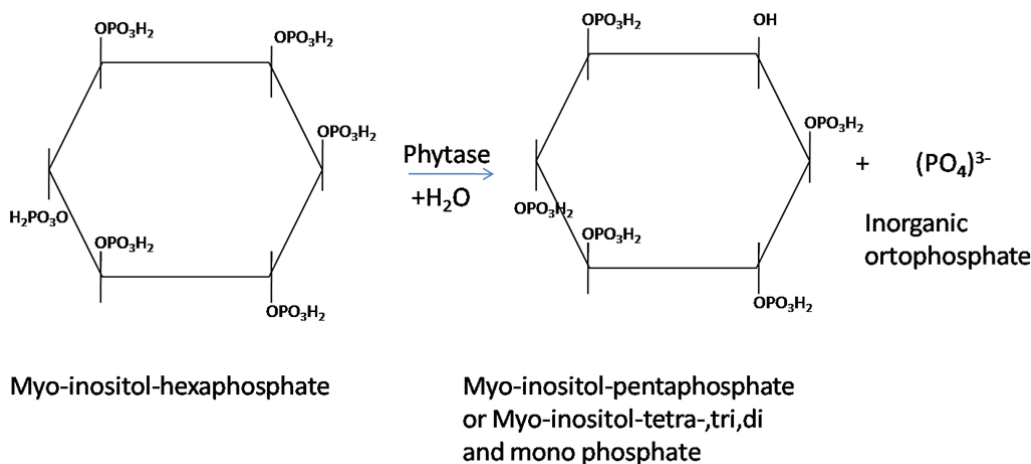


Figure 2.2 General enzymatic reaction of phytase liberating inorganic orthophosphate (Adapted from Liu *et al.*, 1999)

Different phytase enzymes degrade phytate at different rates and to different extents. For example, after Wyss *et al.* (1999) incubated phytic acid with *Aspergillus niger* phytase (Natuphos), 80% of the phytic acid was degraded to IP1 and 20% was degraded to IP2. *Escherichia coli* phytase degraded phytic acid to 78% IP2, 15% IP3, 5% IP1 and 2% IP4. However, by using excess phytase, the end product of bacterial and fungal phytases is always myo-inositol 2-monophosphate (Wyss *et al.*, 1999). Phytase efficiency may be affected by the matrix surrounding the phytate (Brejnholt *et al.*, 2011) and phytate dephosphorylation is therefore dependant on the feed source. HiPhos phytase is able to degrade 83% of the IP6+IP5 in maize soya bean meal, 78% in wheat and only 52% in soya bean meal (Brejnholt *et al.*, 2011).

The hydrolysis of phytate to lower inositol phosphates, which are rather innocuous, decreases the anti-nutritive and chelating properties of phytate in a disproportionate manner (Luttrell, 1993; Selle *et al.*, 2011). The negative effects inositol phosphates had on Ca and Zn bioavailability in rats were much more pronounced for phytate than that of lower inositol phosphates. No negative effects on Zn and Ca availability were observed when four or fewer sites on the inositol were phosphorylated (Lonnerdal *et al.*, 1989). Lonnerdal *et al.* (1989) hypothesised that dephosphorylation of phosphate groups from phytate decreases its mineral binding strength and increases its solubility. Another possibility is that the complexing capacity of inositol may be affected by the configuration of the phosphate groups (Lonnerdal *et al.*, 1989), possibly affecting the efficiencies of 3- and 6- phytases. Unfortunately phytate dephosphorylation is a stepwise process and considerable amounts of intact phytate (IP6) can still occur in the ileum (Selle *et al.*, 2011).

The minimization of the anti-nutritive effects of phytate together with the P equivalence of phytases are dependent on the rate and extent of phytate hydrolysis (Selle *et al.*, 2011). Phytase sequentially dephosphorylates the inositol ring, but the rate of hydrolysis decreases as dephosphorylation progresses (Greiner *et al.*, 1993). The decreased rate in hydrolysis is likely to be due to phytase inhibition from the released orthophosphate and an inherently lower hydrolysis rate of the lower molecular weight intermediates (Greiner *et al.*, 1993). Certain phytases hydrolyse the “pool” of higher molecular weight inositol phosphates prior to the lower molecular weight intermediates and therefore higher levels of phytase might allow dephosphorylation of all the intermediates to occur more effectively in the gastrointestinal tract of the animal (Cowieson *et al.*, 2006).

If described on the basis of substrate specificity, there are two classes of phytases, phytases with broad substrate specificity (*Emericella nidulans*, *Myceliophthora thermophila* and *Aspergillus fumigatus*) and those that are specific for phytate (*A. niger*, *E. coli* and *A. terreus*) (Wyss *et al.*, 1998). The broad substrate specific enzymes readily degrades to myo-inositol 2-monophosphate with only a small amount of accumulation of intermediates, whereas phytases that are specific for phytate do not hydrolyse the intermediates (lower inositol phosphates) as effectively as they do phytate. Initially these two classes of phytase release P at the same rate if the initial phytase activity is the same, but with time the rate of phosphate liberation and the amount of P released is higher for the phytases with broad substrate specificity. Unfortunately phytases with broad substrate specificity have a lower specific activity for phytate than the other class of phytases but they release all five phosphate groups

of phytate more readily and are therefore better suited for animal nutrition purposes (Wyss *et al.*, 1998).

Only a few studies have investigated the dephosphorylation of phytate in the gastrointestinal tract. Camden (2001) reported a phytate degradation of 35% when broilers were supplemented with 500FTU *A. niger* phytases, while Van der Klis *et al.* (1997) reported 58% degradation in layers when supplemented with the same level of *A. niger* phytase. Selle *et al.* (2011) suggested phytase being more effective in layers due to the longer digestion times in the fore-stomach and lower levels of phytase can thus be included in the diets of layers.

2.3.2 Initiation site of phytate dephosphorylation

There are three classes of phytases (3-, 5-, 6-phytase) classified by the International Union of Pure and Applied Chemistry and the International Union of Biochemistry (Greiner & Konietzny, 2011). These enzymes are named according to their dephosphorylation initiation site. The 3-phytase (EC 3.1.3.8) also known by its systematic name, myo-inositol-hexakisphosphate 3-phosphohydrolase, initiates phytate dephosphorylation at the D-3 phosphate ester bond of phytate. The other microbial phytase used in animal feed, 6-phytase (3.1.3.26), first removes the phosphate residue at the D-6 (L-4) position (Van der Kaay & Van Haastert, 1995; Greiner *et al.*, 2000; Lassen *et al.*, 2001). Phytases from plant origin are categorized under 6-phytases, but they initiate hydrolysis at the L-6 (D-4) position (Brinch-Pedersen *et al.*, 2003) and according to the current rule to number myo-inositol phosphates in the D configuration (counter clockwise) these plant phytases should actually be classified as a 4-phytase (Greiner & Konietzny, 2011). It is still unclear if the site where a phytase initiates dephosphorylation has an effect on its efficiency (Greiner & Konietzny, 2011).

2.3.3 Phytase activity

Phytase activity is measured by mixing the enzyme with the substrate and determining how fast the substrate is converted to the end product. The rate at which orthophosphate is hydrolysed from phytate under controlled conditions measures phytase activity. Phytase activity is expressed in units that define the number of reactions occurring each minute under specific assay conditions. A unit of phytase activity (FTU) can be defined as the amount of enzyme that catalyses the release of 1 μmol inorganic orthophosphate per minute from 0.0051 mol/L sodium phytate at pH 5.5 and temperature of 37 °C (Engelen *et al.*, 1993). The definition is a useful measurement of phytase activity under assay conditions (Selle & Ravindran, 2007). Unfortunately, a standard unit for the measurement of phytase activity unit does not exist, which creates confusion in the feed industry (Selle & Ravindran, 2007). Different manufacturers use similar *in vitro* conditions when determining phytase activity, but the buffer (acetate or citrate), extraction time, incubation time and sample size may differ. Therefore different abbreviation for phytase activity (FTU, FYT, U) exist. It is important to keep in mind that different manufacturers use different procedures and the same unit definition, therefore the values are method dependant. It is important to measure the *in vitro* phytase activity for labelling purposes, even though the *in vivo* bio-activity of the phytase differs from the *in vitro* measurement.

Difficulty exists when comparing the P releasing efficiency of commercial phytase enzymes. By using the standard assay, the quantity of each phytase product that needs to be added to the diets to attain equal phytase activity can be determined. Measurement of specific activity of a phytase is dependent on the release of inorganic P from sodium phytate at pH 5.5 but pH optima for phytase activity differs between commercial enzymes. Therefore the P releasing ability of enzymes with pH optima resembling gut pH may be underestimated and instead of adding the quantity of each enzyme that will attain equal phytase activity, the specific activity in the gut might be higher for certain enzymes (Augsburger *et al.*, 2003).

2.3.4 Nutrient releasing abilities of phytase

2.3.4.1 Effects of phytase on phosphorus and mineral availability

As mentioned in section 2.2.2, phytate has the ability to bind Ca and other minerals, forming insoluble salts and rendering these minerals unavailable for absorption. Phytase enzymes have the ability to hydrolyse these bonds and release minerals from the insoluble salts, consequently increasing the bioavailability of the minerals (Kornegay *et al.*, 1996). The beneficial effects of exogenous phytase might be due to the release of macro and micro minerals from phytate-mineral complexes (Brenes *et al.*, 2003).

Dietary P and phytate levels may influence the efficiency of phytases. Ravindran *et al.* (2000) supplemented exogenous phytase to broiler diets with low non phytate phosphorus (npP) and varying phytic acid levels. The authors reported a 40.3 to 58.9% increase in the digestibility of phytate phosphorus together with a 21 to 28 percentage unit increase in ileal phosphorus digestibility when phytase was supplemented to diets low in P (npP = 0.23 g/kg) with phytic acid levels of 10.4 or 15.7 g/kg, respectively. Phosphorus digestibility, however, decreased when diets supplemented with phytase had adequate npP levels. By lowering the npP levels in broiler diets from 3.8 to 2.2 g/kg, or by supplementing HiPhos (DSM Nutritional Products, Basel, Switzerland) phytase to maize soya bean diets with 2.2 g/kg npP, Shaw *et al.* (2011) noticed that the P levels in the excreta decreased with 25 percentage units and 42 percentage units, respectively. Selle & Ravindran (2007) summarised the P equivalency of several phytase enzymes in several studies. Different results were obtained for different enzymes, but collectively the studies indicate that 805 FTU phytase activity is equivalent to 1.05 g/kg inorganic P.

Ravindran *et al.* (2006) measured the apparent ileal digestibility of certain minerals in broilers fed maize soya bean diets supplemented with phytase. Phytase addition did not increase apparent ileal digestibility of K, Fe or Zn, but did increase the apparent ileal digestibility of Mg, Cu, Na, Mn P and Ca. Whereas Cowieson *et al.* (2006) noticed an increased retention for Mg, Cu, Na, P, K, Fe and S but no improvement in Ca and Mn retention was observed when phytase was added to maize soya bean based broiler diets. Brenes *et al.* (2003) reported a linear increase in Ca, P and Zn retention when broiler diets were supplemented with graded levels of Natuphos (BASF, Ludwigshafen, Germany) ranging between 0 to 600 FTU. Retention of Ca, P and Zn in broilers increased with by up

to 9, 10 and 16% respectively. Numerous studies reported an increase in Ca retention when diets were supplemented with phytase, but the Ca equivalencies in these studies were inconsistent (Mitchell & Edwards, 1996; Augspurger & Baker, 2004; Yan *et al.*, 2006).

A ratio of approximately 2 Ca to 1 npP (weight/weight) is appropriate for broilers (NRC, 1994). In order to have optimum growth performances in broilers it is important to maintain the ideal Ca:aP ratio (NRC, 1994) and therefore diets supplemented with phytase must be corrected for Ca to maintain Ca:aP levels. When diets contains a low level of P but a normal level of Ca, decreased feed intake and growth can be expected. If however the levels of both these minerals are low, it would prevent the depression in feed intake (Sebastian *et al.*, 1997).

2.3.4.2 Effect of phytase on protein and amino acid digestibility

Hydrolysis of the ester bonds in phytate most likely releases phytate-bound proteins, consequently increasing the bioavailability of dietary protein. Furthermore, phytase supplementation reduces phytate levels in the diet and may therefore enhance amino acid digestibility by reducing the inhibitory effects phytate has on proteases (Sebastian *et al.*, 1997). Since phytase enzymes are rather expensive to add to poultry or swine diets, improvements in protein utilization will increase the cost effectiveness of phytases (Peter & Baker, 2001).

Phytase supplementation increased apparent and true ileal digestibility of nitrogen (N) and amino acids in turkeys, but results were influenced by dietary npP levels (Yi *et al.*, 1996). Together with npP levels, dietary ingredients and Ca concentrations can also influence the results (Ravindran *et al.*, 2000; Sebastian *et al.*, 1997). Furthermore, phytase supplementation does not increase the digestibility of all the amino acids and the magnitude of the response varies depending on the amino acid considered (Ravindran *et al.*, 2000). Sebastian *et al.* (1997) reported that phytase supplementation in female broilers increased apparent ileal digestibility of almost all the essential and non-essential amino acids (except for methionine, phenylalanine, lysine, and proline) but only increased the apparent ileal digestibility of methionine and phenylalanine in male broilers. However in studies reported by Ravindran *et al.* (2000), phytase supplementation of diets consisting of wheat, sorghum and soya bean meal increased ileal nitrogen digestibility and the digestibility of all the essential amino acids (methionine was not tested) in male broiler diets. It is important to note, even though apparent ileal digestibility was increased in the studies of Sebastian *et al.* (1997), only feed intake and body weight increased whilst the feed conversion ratio (FCR) was unaffected.

The addition of phytase in conjunction with phytate reduces the excretion of endogenous amino acids and minerals (Cowieson *et al.*, 2004). It is expected that with the increase in amino acid and protein digestibility through the addition of phytase, there will also be an increase in protein utilization when measured by the growth-assay methodology (gain per unit of protein intake or crude protein accretion/protein intake). However, this is not always the case; neutral results rather than positive results are mostly obtained (Peter & Baker, 2001; Augspurger & Baker, 2004).

When soya products are fed to monogastric animals, the protein are first limiting in the sulphur containing amino acids (cysteine and methionine) and second limiting in threonine (Fernandez *et al.*, 1994). Peter & Baker (2001) suggested that when the protein digestion and utilization improved where chickens were fed only soya bean meal as a protein source, this would be due to the increased utilization of the sulphur amino acids. However, due to the lack of increased chick performance when phytase was supplemented to protein deficient diets with methionine and cysteine being first limiting; and an increase in performance in chicks receiving the diet with added cysteine and methionine, Peter & Baker (2001) concluded that phytase supplementation does not improve the utilization of cysteine and methionine.

Sodium excretion usually increases when Na binds to phytate, but phytase supplementation can decrease this effect (Ravindran *et al.*, 2006) and may therefore influence the energy utilization in animals (Selle & Ravindran, 2007). High levels of phytate increases Na excretion and has an influence on the Na status of chickens, consequently affecting the acid-base homeostasis of the bird. The mechanism involved in the absorption of glucose and other amino acids are Na-dependant co-transport mechanisms and it is therefore possible that phytate can compromise the uptake of these nutrients (Selle & Ravindran, 2007). The dietary electrolyte balance (DEB) in a diet can be calculated as $\text{Na}^+ + \text{K}^+ - \text{Cl}^-$ and should be 250 meq/kg for optimal growth and litter quality in broilers. The DEB of a diet can have an influence on the response phytase has on amino acid digestibility (Haydon & West, 1990). Furthermore, an increased DEB has been shown to increase amino acid digestibility (Haydon & West, 1990).. Therefore the improvement in amino acid absorption from phytase supplementation may be due to the increased Na availability for the Na^+ dependant transport system (Selle & Ravindran, 2007). Phytase is more likely to enhance amino acid digestibility when the DEB levels of the diet is low (Ravindran *et al.*, 2008). This may be a contributing factor to the varying phytase responses in amino acid digestibility assays reported in the literature.

2.3.4.3 Effect of phytase on apparent metabolisable energy (AME)

Phytase supplementation may increase the AME of broiler diets (Ravindran *et al.*, 1999; Ravindran *et al.*, 2000; Ravindran *et al.*, 2006; Santos *et al.*, 2008). Selle & Ravindran (2007) revised 12 papers and on average, phytase supplementation increased the AME with 0.36 MJ/kg (2.8%). Phytase supplementation can increase the AME up to 5.7%, depending on the level of dietary npP. Responses are greater in diets with low available P (aP) levels compared to diets with adequate aP levels (Ravindran *et al.*, 2000). The increase in energy utilization is most probably due to an increase in protein, starch and fat digestibility (Selle & Ravindran, 2007)

2.3.5 Effect of exogenous phytase on production parameters

The effect of phytase on intake and growth performance has been widely investigated and the results are mostly positive. It has been shown that phytase supplementation in broiler diets can increase feed intake and body weight gain of chickens receiving diets with low levels of inorganic P in order to achieve the same body weight as chickens receiving diets high in inorganic P (Kornegay *et al.*, 1996;

Zhang *et al.*, 1999; Johnston & Southern, 2000; Ravindran *et al.*, 2008; Aureli *et al.*, 2011; Shaw *et al.*, 2011). Unfortunately the feed efficiency responses are not as consistent. Some studies noted an improvement in feed conversion ratio (Shirley & Edwards, 2003; Ravindran *et al.*, 2008; Aureli *et al.*, 2011; Pirgozliev *et al.*, 2011; Shaw *et al.*, 2011) whereas others did not find any significant improvements when phytase was added to diets containing low npP levels (Zhang *et al.*, 1999; Johnston & Southern, 2000; Shaw *et al.*, 2010). However in the case of Shirley & Edwards (2003), Aureli *et al.* (2011), Pirgozliev *et al.* (2011) and Shaw *et al.* (2011), significant improvement of FCR was dependent on phytase levels. It has been established that aP can influence weight gain and feed intake. A decrease in aP content in the diet from 3.5 to 2.5 g/kg can decrease weight gain by 6% and feed intake by 3% (Brenes *et al.*, 2003). However, weight gain and feed intake can increase quadratically by means of phytase supplementation (Brenes *et al.*, 2003).

It is expected that increased inclusion rates of phytase will increase phytate degradation and improve the magnitude of response to phytase. Shirley & Edwards (2003) demonstrated that tP retention and phytate-P disappearance responded quadratically when graded levels (93.75 to 12000 FTU) of Natuphos (BASF, Ludwigshafen, Germany) were supplemented to maize soya bean broiler diets (2.72 g/kg phytate-P/kg, 4.6 g/kg tP), but there was no statistical differences for live weight gain, feed intake or feed efficiency between chickens receiving the control diet or diets containing 1500 to 12000 FTU Natuphos phytase.

Ravindran *et al.* (2001) also supplemented broiler diets containing higher levels of tP (3g phytate P/kg; 7.5 g tP/kg) with seven different levels of Natuphos ranging from 0 to 1000 FTU. In contrast to findings of Shirley & Edwards (2003), weight gain reached a plateau at 500 FTU. Furthermore, Kornegay *et al.* (1996) illustrated that growth responses to phytase supplementation decreased with increased dietary npP and tP levels. The maximum growth response in broilers receiving diets with 2.0, 2.7 or 3.4 npP/kg occurred when diets were supplemented with 1000, 800 and 600 FTU phytase, respectively. Improvement in FCR only occurred in broilers receiving diets with 2.0 g npP/kg. The data suggests that the magnitude of the response due to increased phytase levels may decrease if the tP level in the diet is high (Selle & Ravindran, 2007). There are two possible explanations to this phenomenon (i): orthophosphate, the end product of phytase dephosphorylation, may inhibit phytase activity or (ii): the increased release of P as a result of higher levels of phytase may alter the Ca:P in the gastrointestinal tract (Selle & Ravindran, 2007).

The nutrient specifications of a diet can alter the effects phytase has on growth performance. Phytase supplementation in diets with reduced Ca, P, protein/amino acids and ME has more robust effects on growth and FCR than supplementation to standard diets (Selle *et al.*, 1999). Furthermore, phytase supplementation has the potential to increase weight gain of chickens fed on modified diets with reduced nutrients in order to compare with chickens raised on standard diets (Selle *et al.*, 1999). If nutrient specifications are decreased appropriately, microbial phytase has the potential to decrease the cost of live weight gain if supplemented to low cost modified diets (Selle *et al.*, 1999).

2.3.6 Effect of phytase on bone parameters

Bone status is very important in poultry production and can be used as an indicator of mineral adequacy in the diet. Calcium, P and Mg are the three macro-minerals that form the structural components of the skeleton. About 99% of the Ca in the animal body is in the skeleton (Meyer *et al.*, 1983). Bone is a metabolically active tissue with continuous turnover and remodelling activity (Meyer *et al.*, 1983). The Ca:P ratio in the bone is approximately 2:1 and is primarily in the form of hydroxyapatite crystals; $[\text{Ca}_{10}^{2+} \times (\text{PO}_4)_6 (\text{OH})_2 (\text{H}_3\text{O}^+)]$ (Pond *et al.*, 2005).

The formation of hydroxyapatite crystal for skeleton ossification requires that the product of Ca ions and P ions should exceed a critical minimum level in the fluid surrounding the bone matrix (Swatland, 1994). A certain concentration of Ca^{2+} and PO_4^{2-} is required to precipitate CaPO_4 in the crystal lattice structure. If either one or both of the minerals falls below the required concentration, ossification fails to occur (Meyer *et al.*, 1983; Pond *et al.*, 2005). If plasma concentration of Ca is low, Ca absorption from the gastrointestinal tract and Ca resorption from the bone increases, but high plasma Ca levels inhibits Ca resorption from the bone (Pond *et al.*, 2005). When P in the diet is in excess, it has the same effect on the skeleton as when Ca deficiency occurs. A reduction in bone ash can be noted when there is a deficiency in dietary Ca or when there is an Ca:P imbalance (Pond *et al.*, 2005; Swatland, 1994).

The degree of bone mineralisation affects bone strength and P or Ca deficiency can increase bone breakage and defects (Brenes *et al.*, 2003). Defects or breakage of the tibia and femur during processing results in downgrading of the meat. Fracturing of the clavicle bones can cause bloody breast meat or bloodiness on the *pectoralis minor* muscle, also known as the tenderloin (Driver *et al.*, 2006). Deformity of the metatarsi affects the birds walking ability and will therefore affect feed intake and production (Orban *et al.*, 1999). The release and bioavailability of phytate-P through the release of phytase can be evaluated by responses in live weight gain and bone development. Live weight performance and mineral retention are good indicators of dietary change, but bone mineral concentrations are generally better indicators of P status and are more accurate in determining P bioavailability of the diet (Brenes *et al.*, 2003). In addition to bone ash, bone breaking strength, bone weight and bone volume can be used to evaluate bone mineralisation in poultry (Onyango *et al.*, 2003).

Brenes *et al.* (2003) showed that by lowering the P content of a diet by 1 g (3.5 to 2.5g/kg), tibia ash can decrease by 1%. Calcium and P concentrations in the tibia ash did however increase, but Zn concentrations decreased. Phytase supplementation has the ability to increase the tibia's ash content by up to 4% by increasing the Ca, P and Zn content in the bone (Brenes *et al.*, 2003).

Several trials have determined the effects commercial phytase enzymes have on bone parameters (Table 2.2). Bone ash percentage and tibia breaking strength decrease with a reduction in dietary npP levels, most probably due to the lack of P available for mineralisation in the body and bone development. Supplementing diets low in npP with different levels of HiPhos (500 to 2000 FYT/kg diet) increased tibia ash percentage by 6 to 14% (Aureli *et al.*, 2011; Shaw *et al.*, 2011). Ash

percentage and bone breaking strength is known to improve with phytase level increments (Shaw *et al.*, 2011). Supplementing low npP diets with HiPhos, Ronozyme or Phyzyme has the ability to increase tibia breaking strength (Shaw *et al.*, 2010).

Percentage tibia ash has a high negative correlation with broken tibias, broken femurs, broken clavicles and bloody *pectoralis minor* muscles (Driver *et al.*, 2006). Broiler chickens raised on starter diets with 6.0 g/kg Ca and 2.4 g/kg npP (4.7 g/kg tP), and on grower diets with 3.0 g/kg Ca and 1.3 g/kg npP (3.7 g/kg tP) had 16.3% broken tibia incidence and 6.3% broken femur incidences during processing. However, supplementing the diets with phytase and 1 α -hydroxycholecalciferol decreased the incidence of tibia breakage to zero and femur breakage to 1.3% (Driver *et al.*, 2006).

Table 2.2 Effect of commercial phytases on bone parameters of broilers

Phytase	Activity (FTU)	npP		tP		Ca		Bone strength (N)			Tibia ash %			Age (days)	Reference
		PC	NC	PC	NC	PC	NC	PC	NC	NC+Phy	PC	NC	NC+Phy		
HiPhos	500	0.38	0.22	0.67	0.49	0.94	0.94	142	72 *	107 *#	39	29 *	35 *#	21	Shaw <i>et al.</i> , 2011
HiPhos	1000	0.38	0.22	0.67	0.49	0.94	0.94	142	72 *	120 *#	39	29 *	38 *#	21	Shaw <i>et al.</i> , 2011
HiPhos	2000	0.38	0.22	0.67	0.49	0.94	0.94	142	72 *	131 #	39	29 *	39 #	21	Shaw <i>et al.</i> , 2011
HiPhos	1000	-	-	0.56	0.41	0.60	0.60	172	74 *	197 *#	48	37 *	50 *#	22	Aureli <i>et al.</i> , 2011
HiPhos	2000	-	-	0.56	0.41	0.60	0.60	172	74 *	221 *#	48	37 *	51 *#	22	Aureli <i>et al.</i> , 2011
Phyzyme	500	0.45	0.25	0.68	0.55	1.0	1.0	178	40 *	101 *#	-	-	-	21	Shaw <i>et al.</i> , 2010
Phyzyme	500	0.35	0.25	0.78	0.55	1.0	1.0	189	40 *	101 #	-	-	-	21	Shaw <i>et al.</i> , 2010
Phyzyme	500	0.45	0.25	0.78	0.55	1.0	1.0	282	90 *	182 #	-	-	-	28	Shaw <i>et al.</i> , 2010
Ronozyme	750	0.45	0.25	0.78	0.55	1.0	1.0	282	90 *	252 #	-	-	-	28	Shaw <i>et al.</i> , 2010

npP: non phytate phosphorus; tP: total phosphorus; PC: positive control diet; NC: negative control diet; NC+Phy: negative control diet supplemented with phytase

* Means significantly differed from the positive control ($P < 0.05$)

Means significantly differed from the negative control ($P < 0.05$)

2.3.7 Matrix values of phytase

In addition to the P and Ca releasing abilities of phytase, these enzymes are capable of releasing proteins and increase the energy of a diet; a phenomenon also known as the 'extra phosphoric effects' of phytase (as discussed in 2.3.3). Some nutritionists add matrix values for phytase in order to benefit from the use of phytase supplementation. Matrix values indicates the amount of extra nutrients (amino acids, ME, P, Ca) that will be available for the animal for absorption when phytase is added to their diet (Shelton *et al.*, 2004). When matrix values are used, diets can be formulated with a lower amount of Ca (limestone), P (monocalcium phosphate), crystalline amino acids and ME, subsequently reducing the cost of feed (Shelton *et al.*, 2004).

2.3.8 Effect of phytase on water intake

It is speculated that phytase supplementation in animal diets might increase water intake and an increase in water intake might increase litter moisture which in turn will have a negative effect on litter quality. Phytase supplementation increases mineral availability and decreases endogenous mineral secretions in chickens (Cowieson *et al.*, 2004; Cowieson *et al.*, 2006). As a result, osmolality within the gastrointestinal tract of the chicken will rise and changes in acid-base homeostasis will take place. This phenomenon might increase the demand for water to maintain homeostasis (Cowieson *et al.*, 2004). However, Cowieson *et al.* (2004) suggested that if nutritionists take the reduction in endogenous mineral loss into consideration when formulating broiler diets with phytase, water intake might not increase. Ravindran *et al.* (2008) demonstrated that phytase supplementation does not affect the quality (moisture and stickiness) of litter. Nevertheless, DEB levels higher than 300 meq/kg reduced the dry matter in the excreta and increased the stickiness of the excreta. The authors concluded that the negative effect on litter quality was most likely due to increased Na levels, which in turn may lead to increased water consumption and water excretion. An increase in water intake has been noted in chickens supplemented with phytase enzymes (Debicki-Garnier & Hruby, 2003), however, published papers on the effect phytase has on water intake in broilers are scarce.

2.3.9 Effects of phytase on the duodenum of broilers

The most important function of the gastrointestinal tract is nutrient, water and electrolyte transport. The small intestine can be divided into three sections: the duodenum, the jejunum and the ileum. The surface of the small intestine contains finger-like projections known as villi, which increases the surface area of the small intestine and therefore increases its absorptive capacity (Silverthorn, 2007). At the base of the villi, tubular invaginations known as crypts extend down the connective tissue (Silverthorn, 2007). Absorptive cells, known as enterocytes originate from stem cells located within the crypt (Shen, 2009). As they mature, enterocytes migrate upwards towards the tip of the villi, the number of digestive enzymes in the cells increases and consequently absorption increases. When the enterocytes reaches the tip of the villi, they are expelled into the lumen of the intestine (Meyer *et al.*, 1983).

A decrease in villi height has been noted with the reduction in nutrient availability (Pluske *et al.*, 1996; Van Beers-Schreurs *et al.*, 1998) and a positive correlation exists between crypt depth and cell production within the crypt (Hedemann *et al.*, 2003). Furthermore, nutrients within the lumen of the small intestine stimulate cell proliferation (Goodlad & Wright, 1984). The crypts are responsible for the cells in the villi and the deeper the crypts, the quicker the tissue turnover in order to permit villus renewal (Awad *et al.*, 2009). However, shorter villi and deeper crypts may decrease nutrient absorption, increase endogenous losses through increased loss of enterocytes and as a result decrease production performance of the animal (Xu *et al.*, 2003). Therefore the villus height: crypt depth ratio can be used as an indicator of possible digestive capacity of the small intestine and a decreased ratio usually results in a decreased digestion and absorption capacity (Montagne *et al.*, 2003). Villi atrophy reduces the absorptive surface of the small intestine. Moreover, the cells usually lost are the mature enterocytes, thereby decreasing absorption even more (Montagne *et al.*, 2003). Phytate decreases nutrient availability and might therefore have an effect on villus height and crypt depth. The effect of phytase on intestinal morphology is scarce. A few trials have been done on the effect of phytase together with organic acids on small intestinal morphology (Khodambashi-Emami *et al.*, 2013; Smulikowska *et al.*, 2010).

Organic acids are capable of modifying gut microflora and might therefore have a beneficial effect on immunity and gut health (Khodambashi-Emami *et al.*, 2013). Supplementing broilers with organic acids decreases the pH in the lumen and consequently accelerates the conversion of pepsinogen to pepsin. This phenomenon decreases the formation of insoluble mineral complexes and as a result improves the absorption rate of amino acids and minerals (Park *et al.*, 2009). In addition, it is speculated that organic acids can enhance microbial phytases with a low pH optimum by decreasing the pH in the gastrointestinal tract.

Khodambashi-Emami *et al.* (2013) reported that phytase or organic acid supplementation to maize-soya bean based diets low in aP, increased duodenum and jejunum villi height and the villi height: crypt depth ratio. Phytase or organic acid supplementation did not affect duodenum crypt depth, but low aP diets resulted in an increased jejunum crypt depth compared to diets supplemented with phytase. Unexpectedly, a combination of phytase and organic acid supplementation did not result in an additive or synergistic effect on gut morphology. However, in the studies reported by Nourmohammadi & Afzali (2013), phytase supplementation increased crypt depth and decreased villus height: crypt depth ratio. Nourmohammadi & Afzali, (2013) also reported an increase in villi height, crypt depth, villus height: crypt depth ratio and villi width in broilers supplemented with citric acid compared to the control birds. However, there was no interaction between phytase and citric acid supplementation compared to diets with normal P levels.

On the other hand, Smulikowska *et al.* (2010) reported shorter villi heights and deeper crypt depths in chickens fed wheat, soya bean and rapeseed based diets low in npP compared to chickens receiving normal P levels. Furthermore, organic acids decreased jejunum villi heights, but phytase supplementation was able to increase villi heights and crypt depths. In the study of Smulikowska *et al.* (2010), jejunum villi height in chickens fed wheat, soya bean and rapeseed based diets low in npP

were shorter and crypt depth were deeper compared to diets with normal P levels; organic acids further depressed jejunum villi heights, but phytase supplementation was able to increase the villi heights and crypt depths. Wu *et al.* (2004) also observed an increase in villi height in the duodenum of broilers supplemented with phytase. The authors speculated that the effects exogenous phytase may have on the gastro intestinal tract morphology of broilers may contribute to the improved performance normally observed when broiler diets are supplemented with phytase.

2.3.10 Effects of phytase on organs and immune function

Nutritionists usually only considers production parameters when choosing ingredients for broiler diet formulation. However, the nutrient profile and the balance of nutrients can have a substantial effect on the immune system of the animal (Ghahri *et al.*, 2012). The well-known trace minerals that play a role in immunity function are: Zn, Se, Mn and Cu (Kidd, 2004). As discussed in 2.3.3.1, phytase has the ability to increase the digestibility or retention of Zn (Brenes *et al.*, 2003), Mn and Cu (Cowieson *et al.*, 2006). Therefore phytase might have an effect on the immune status of broilers.

Furthermore, phytase supplementation has been shown to decrease sialic acid content in the excreta, which is an indicator of endogenous secretions such as mucin (Pirgozliev *et al.*, 2005). High concentrations of sialic acid are often an indicator of certain health problems such as bacterial infections, cellular senescence and pathological conditions. Therefore a reduction of sialic acid in excreta of phytase supplemented broilers might be an indication that phytase benefits the health of broilers (Pirgozliev *et al.*, 2005) and can therefore be one of the mechanisms that contributes to the mode of action of phytase.

In addition, Zyla *et al.* (2000a) reported a significant increase in bursa weights in 21 day old broilers supplemented with phytase, together with acid phosphatase, pectinase and citric acid. Stem cells proliferate and differentiate in the bursa to become B-lymphocytes, which in turn are responsible for antibody production during an immune response. As a result, the positive influence phytase normally has on growth can be the result of the nutrient releasing effect of phytase together with a physiological regulation mechanism (Ghahri *et al.*, 2012). Phytate may cause inflammation in the gastrointestinal tract by means of direct irritations or by enhancing the growth of intestinal microflora. The activation of the immune system is an energy demanding process and therefore phytase supplementation and the subsequent hydrolysis of phytate might decrease the energy wasted through unnecessary immune reactions (Ghahri *et al.*, 2012).

2.4 Commercial phytase enzymes

Even though the enhancement of phytate bound phosphorus and bone mineralisation in chickens through the use of exogenous phytase enzymes was demonstrated five decades ago (Warden & Schaible, 1962), the first successful commercial phytase (Natuphos®) was only developed in 1991 by BASF.

For many years, Natuphos was the only phytase enzyme in the market (Simon & Igbasan, 2002). It is a 3-phytase produced by the fungus *Aspergillus*. In order to produce this enzyme on large scale, BASF genetically modified the recombinant *A. niger* strain with the *A. ficuum* phytase gene (Zhang *et al.*, 2000). DSM marketed Ronozyme, a 6-phytase produced by the *Aspergillus oryzae* strain, transformed with a gene from *Peniophora lycii* (Simon & Igbasan, 2002). Phyzyme is classified as a 6-phytase and is produced by *Schizosaccharomyces pombe* and modified with the AppA gene from *E. coli* (Kerr *et al.*, 2010). DSM was the first company to use synthetic genes in a phytase product. Ronozyme HiPhos is a 6-phytase (histidine acid phosphatase phytase), produced by *Aspergillus oryzae* and contains two synthetic genes that mimics a phytase gene from *Citrobacter braakii* ATCC 51113 (Guggenbuhl *et al.*, 2012).

All types of commercially available phytases belong to the class of histidine acid phytases (based on the catalytic mechanism). Acid phytases show a maximum phytate dephosphorylation at pH 5, therefore histidine acid phytases are suspected to be most efficient in the crop (pH 4.0 to 5.0) or in the proventriculus and gizzard (pH 2.0 to 5.0) of the chicken (Greiner & Konietzny, 2011). The classes of certain commercial enzymes with its production strain and gene origin are summarised in Table 2.3. Currently there are quite a few phytase enzymes available commercially. However, it is quite difficult to effectively compare them. The recommended dosages to release similar amounts of phytate bound P differs greatly among different phytase sources.

Table 2.3 Production strain and gene origin of commercial enzymes

Name of Phytase	Production strain/organism	Origin of phytase gene	Class	Company	Reference
Natuphos	<i>Aspergillus niger</i>	<i>Aspergillus ficuum</i>	3-phytase	BASF	Zhang <i>et al.</i> , 2000
Phyzyme	<i>Schizosaccharomyces pombe</i>	AppA gene from <i>E. coli</i>	6-phytase	Danisco	Kerr <i>et al.</i> , 2010
Ronozyme	<i>Aspergillus oryzae</i>	<i>Peniophora lycii</i>	6-phytase	DSM	Simon & Igbasan, 2002; Kerr <i>et al.</i> , 2010
HiPhos	<i>Aspergillus oryzae</i>	<i>Citrobacter braakii</i>	6-phytase	DSM	Guggenbuhl <i>et al.</i> , 2012

2.4.1 Factors affecting the efficiency of commercial phytases

Many factors influence the efficiency of phytase enzymes. For commercial phytases to be effective, the enzymes should effectively release phytate-P, maintain activity after being exposed to the harsh conditions of feed processing, should be stable in the acid environment of the crop and proventriculus, be resistant to inactivation by proteolytic enzymes and must be cheap to produce (Igbasan *et al.*, 2000; Greiner & Farouk, 2007). Depending on the source of the phytase gene and the micro-organism from which the enzyme is produced, phytases exhibit different properties relating to

pH and temperature optimum, stability and protease resistance, which can affect the *in vivo* bioefficacy of phytase.

2.4.1.1 Dietary calcium and phytate levels

Dietary phytate and Ca levels are two factors affecting the outcome of phytase trials and contribute to the ambiguous outcomes from trials involving microbial phytases. Mineral-phytate complexes, especially Ca-phytate complexes, reduce phytase activity (Angel *et al.*, 2002). Calcium-phytate complexes are insoluble and resistant to hydrolysis by phytases (Taylor, 1965). Therefore it can be speculated that Ca limits phytate degradation. If phytate hydrolysis takes place before the formation of these complexes, the availability of Ca and phytate bound P will increase (Selle *et al.*, 2009). Presently it is believed that high dietary Ca levels influences the efficiency of microbial phytase. Therefore high dietary Ca levels may be a contributing factor to inconsistency in literature regarding phytase studies (Selle *et al.*, 2009).

Several studies have determined the advantages of low dietary Ca levels and narrow Ca:P ratios on the efficiency of phytase on phytate P utilization. According to Kornegay *et al.* (1996) widening the Ca:tP from 1.1:1 to 2:1 decreases phytase response and efficiency in broilers. Lei *et al.* (1994) reported that increasing the dietary Ca:P ratios from 1.62 to 3.07 decreased weight gain, feed intake and FCR in pigs offered low P, phytase supplemented diets. Aksakal & Bilal (2002) reported an 8.5% increase in P retention when phytase was supplemented to broiler diets with a Ca:P ratio of 2:1, but P retention was increased to 39.8% when the Ca:P ratio was reduced to 1:1. Furthermore, Zyla *et al.* (2000b) reported an 8.4%, 13.4% and 19.6% increase in 21-day weight gain when phytase was supplemented to broiler diets with Ca:P ratios of 1.93, 1.44 and 1.68, respectively. In contrast, Driver *et al.* (2005) reported that phytase supplementation (12000 FTU) was more effective when supplemented to diets with higher Ca levels (8.6 g/kg Ca; 4.5 g/kg tP; 2.4 g/kg npP; Ca:tP = 1.91) with unbalanced Ca:tP ratios than in balanced diets with lower Ca levels (4.7 g/kg Ca; 2.4 g/kg npP; 5.0 g/kg tP; Ca:tP = 0.94). Supplementing phytase to diets high in Ca increased weight gain by 64.5% compared to a 12.5% increase in diets with lower Ca levels. These authors concluded that much of the published papers regarding the effect of Ca:P ratio on phytase efficiency is misleading, reports usually shows an increased performance when phytase is supplemented to narrow Ca:P ratio's, but the margin of improvement in most studies is substantially greater for birds fed diets with wide Ca:P ratios. It was also concluded that the npP levels, together with the absolute concentrations of Ca and P is important in studies regarding phytase efficiency.

Feed ingredients have relatively low Ca levels and therefore Ca-phytate complexes in the diets are limited. Most of the Ca-phytate complexes form in the gastrointestinal tract of chickens (Selle *et al.*, 2009). The formation of insoluble complexes is dependent on the pH and molar ratios of Ca and phytate (Martin & Evans, 1986). Several studies agree that a pH of 5 or higher is essential for the formation of Ca-phytate complexes (Grynspan & Cheryan, 1983; Martin & Evans, 1986; Oberleas & Chan, 1997). However, studies by Graf (1983) and Marini *et al.* (1985) reported that Ca-phytate chelates are capable of forming at relatively low pH levels. Graf (1983) suggested that substantial

amounts of Ca will be bound to phytate in the stomach which may facilitate the precipitation of the complex in the pH environment of the small intestine. The efficacy of exogenous phytases will be influenced by the site where insoluble Ca-phytate complexes form (Selle *et al.*, 2009). Commercial phytases are mostly active in the upper gastrointestinal tract of poultry (Yu *et al.*, 2004). Therefore, if these complexes only form in the pH conditions of the small intestine, the complexes would not impede exogenous phytase activity in the crop, proventriculus or gizzard. But if these Ca-phytate complexes form in acidic conditions, it may affect the efficacy of exogenous phytate supplementation (Selle *et al.*, 2009).

Furthermore limestone, a popular Ca source in monogastric animal diets, has a high acid binding capacity (Lawlor *et al.*, 2005). Thus, Ca supplementation in the form of limestone will tend to increase the pH of the digesta in the gastrointestinal tract. Shafey *et al.* (1991) demonstrated that increasing Ca levels from 10.7 to 25.3 g/kg increased the pH in the crop from 4.89 to 5.32. Therefore high dietary Ca levels, as limestone, will result in a higher digesta pH and increased Ca:phytate molar ratio, both favouring Ca-phytate interactions (Selle *et al.*, 2009). Also, depending on the pH spectrum of the phytase used in the diet, an increase in gut pH due to high levels of limestone (Ca) may negatively influence phytase activity.

It should be kept in mind that phytate is not the only anti nutrient reducing mineral availability. High levels of dietary calcium decrease the availability of other minerals, such as P, Zn Mg and Mn (NRC, 1994). An increase in the crop and ileal pH due to high Ca levels also leads to reduced solubility of mineral complexes (Shafey *et al.*, 1991). The formation of Zn-Ca-phytate complexes can occur in the gastrointestinal tract of animals and is highly insoluble and even more resistant to digestion and absorption (Reddy *et al.*, 1982; Maenz, 2001).

2.4.1.2 The pH profile and pH optimum of microbial phytases

Exogenous phytases added to the diet of broilers are expected to release P during the short time from when the feed is ingested up to the point that feed residues have passed the small intestine. Therefore, their activity should be relevant to the pH encountered in the gastrointestinal tract and it is important that the enzymes are stable under these conditions. The optimum pH, the level of phytase activity at different pH conditions and optimum temperature for different phytase enzymes are shown in Table 2.4. Different phytase enzymes have different pH activity profiles. Even though some phytases show maximal activity in the same pH range, there may be substantial differences in their pH activity profiles. After ingestion, the enzyme passes through the gastrointestinal tract of the animal and the luminal pH of the different sections will determine the enzyme's site of action. The pH in the crop, stomach and small intestine is 4 to 5; 2 to 5 and 6.5 to 7.5, respectively (Simon & Igbanan, 2002). The broadness of the pH profile and the pH optima of the enzyme determine how effective its catalytic activity in the different sections of the gastrointestinal tract will be. It is clear that a broad pH activity profile of a phytase enzyme is advantageous for efficient phytate dephosphorylation in the gastrointestinal tract of monogastric animals.

Table 2.4 *In vitro* properties of phytases from various microbial origins

Phytase origin	Commercial name	pH range (activity %)	Optimum pH	Optimum temperature (°C)	Reference
	Natuphos	2.5 – 6 (50 – 100%)	5.5	50	Igbasan <i>et al.</i> , 2000
	Ronozyme	3.5 – 5.0 (80%)	4.5	50	Igbasan <i>et al.</i> , 2000
	Ronozyme	4.0 – 4.5 (100%)	-	-	Breijnholt <i>et al.</i> , 2011
	Ronozyme	3.0 (35%)	-	-	Breijnholt <i>et al.</i> , 2011
	HiPhos	3.0 – 5.0 (70%)	3.5	-	Breijnholt <i>et al.</i> , 2011
	HiPhos	3.0 – 4.5 (90%)	3.5	-	Breijnholt <i>et al.</i> , 2011
<i>E.coli</i>	-	3.5 – 5.5 (60%)	4.5	60	Igbasan <i>et al.</i> , 2000
<i>Bacillus</i>	-	5.5 – 7.5 (70%)	7.0	60	Igbasan <i>et al.</i> , 2000

If the enzyme has a high optima pH, activity will be at its highest in the small intestine, but then it is important that these enzymes are stable after passing through the stomach (Simon & Igbasan, 2002). The effectiveness of the enzyme is thus determined by its temperature optimum and stability, pH optimum, pH profile and proteolytic stability. Sometimes it is assumed that phytases have to have optimal activity at low pH ranges (2 to 3) in order to act in the stomach and gizzard of pigs and chickens, but the pH from the crop, proventriculus and gizzard increases after feed ingestion (Breijnholt *et al.*, 2011). Brenes *et al.* (2003) supplemented Natuphos phytase together with citric acid to reduce the pH of the crop and expected a synergistic effect on mineral utilization and production. On the contrary, citric acid supplementation negatively affected growth response to phytase. Furthermore, due to different pH optimums and pH profiles of phytases from different origin, the combination of two or more enzymes, especially *Bacillus* and *Aspergillus* phytases, might be beneficial in the hydrolyses of phytate in the entire gastrointestinal tract of the chicken (Park *et al.*, 1999).

As mentioned in 2.3.3, phytase activity and units are measured at standard conditions (pH 5.5, 37 °C, 0.0051 mol/L sodium phytate) therefore its phytate degrading ability will differ under other pH conditions. The phytase units declared do not always represent the amount of P released in the gastrointestinal tract of the animal because the pH conditions in the assays differ from the pH encountered in the different parts of the gastrointestinal tract. If another pH value was used for standard phytase activity determination, a different result would be obtained in respect to ranking phytases (Greiner & Konietzny, 2011). However, phytase bioefficacy is not only determined by its pH activity profiles, but resistance to pepsin and pH stability in the crop and proventriculus is also important (Engelen *et al.*, 1993).

2.4.1.3 Thermostability and temperature optimum of microbial phytases

Temperature also affects enzyme activity. The body temperature of the animal is 38 °C, the optimal activity of fungal and bacterial enzymes ranges from 45 to 60 °C (Igbasan *et al.*, 2000; Simon & Igbasan, 2002) and thus only about 40 to 60% of the maximal enzyme activity will be reached in the gastrointestinal tract of the animal (Igbasan *et al.*, 2000).

Feed pelleting is common practice in poultry nutrition. The enzymes are exposed to temperatures between 60 to 95 °C for short periods during this processing method. There are methods to avoid destruction of enzymes, for example, post pelleting application (spraying the pellets with phytase) or chemical coating of the enzymes. Coating can however influence the release and function of the enzyme in the gastrointestinal tract and spraying the enzymes increases the feed manufacturing cost due to the need of extra equipment and labour (Greiner & Konietzny, 2011). Therefore enzymes with the ability to withstand high temperatures are more suitable as a feed supplement.

Igbasan *et al.* (2000) determined the thermostability of *A. niger*, *P. lycii* and *E. coli* phytase enzymes. All three phytases maintained at least 65% of their activity at processing temperatures of 70 °C and are therefore stable under pelleting conditions of 70 °C. After heating an irreversible conformational change usually takes place in phytases by means of denaturation. Interestingly, Wyss *et al.* (1998) has shown that an *A. fumigatus* phytase has the ability to refold to a fully active conformation after heat exposure for 20 minutes at 90 °C. Only 10% of its activity was lost, thus favouring its use in pelleted feeds.

2.4.1.4 Proteolytic resistance of microbial phytases

A large proportion of supplemented phytase become degraded through the action of proteolytic enzymes (pepsin and trypsin) in the stomach and small intestine of monogastric animals. Therefore high levels of phytase supplementation are necessary in animal diets (Rodriguez *et al.*, 1999). Pepsin is released in the proventriculus and therefore proteolytic resistance of phytases is essential in order to hydrolyse phytate in the proventriculus and the rest of the gastrointestinal tract.

Greiner & Farouk (2007) incubated phytase enzymes with 3000 U pepsin at 37 °C for 30 minutes. After incubation, the remaining activity of phytase derived from *E. coli*, *A. niger* and *P. lycii* was 94.3, 43.1 and 19.6%, respectively. Igbasan *et al.* (2000) also incubated Natuphos, Ronozyme and *E. coli* derived phytases with 2500 to 3500 U of pepsin for one hour at 40 °C. The residual phytase activity after incubation was 25.9, 1.8 and 94.6%, respectively. This also demonstrates that phytases derived from fungi are less resistant to proteolytic enzymes than bacterial phytases.

In addition, Igbasan *et al.* (2000) mixed phytases with digesta from the crop and proventriculus of hens to determine what the proteolytic stability of phytases might be in the gastrointestinal tract. The residual phytase activity for Natuphos, *E. coli* or *Bacillus* derived phytases was greater than 96.5% after mixing the phytase with digesta of the crop (pH 5.02) for 60 min at 40 °C whilst the residual phytase activity after incubation with digesta of the proventriculus (pH 2.75) was between 59 and 70%

for all the phytases except for the *E. coli* derived phytase, which had a residual phytase activity of 92.8%. Phytases are more resistant to proteolysis after incubation in gut digesta compared to direct incubation with proteases (Greiner & Konietzny, 2011). It can be speculated that the substrate phytate in the gut stabilizes the phytase or the presence of proteins in the gut lumen serves as substrates for the proteases. It is important to remember that the proteolytic resistance of the wild type phytase enzymes and the recombinant enzymes derived from these wild-types are not always the same (Greiner & Konietzny, 2011).

The extent to which phytase is degraded is determined by the ratio of protease and phytase. Both Natuphos and a recombinant *E. coli* phytase (r-AppA, EC 3.1.3.2) retained more than 85% of their activity after incubation with trypsin and phytase with a trypsin:phytase ratio of 0.001 or 0.005. When the trypsin concentration was increased to create a trypsin:phytase ratios of 0.01, Natuphos still retained 90% of its activity, but *E. coli* derived phytases retained only 40% of its relative phytase activity. When incubated with pepsin at protease:phytase ratios of 0.002 or 0.005, Natuphos phytase retained 90 and 40% of the original activity, respectively, while the relative phytase activity of the recombinant *E. coli* phytase increased with 10% and 40%, respectively (Rodriguez *et al.*, 1999). Rodriguez *et al.* (1999) concluded that pepsin degrades the recombinant *E. coli* phytase into small peptide segments and as a result, these peptide segments might have a higher phytase activity and have a higher pepsin resistance than the intact phytase protein. However, due to the low activity of exogenous phytases in the neutral pH of the small intestines, the importance of the phytase enzymes reaching the lower parts of the gastrointestinal tract in a more active state is minor. Only *Bacilli* with an optimum pH of 7 will hydrolyse phytate in the small intestine (Igbasan *et al.*, 2000). By means of *in vivo* tests, Pontoppidan *et al.* (2012) showed that HiPhos has the ability to maintain 100% phytase activity in the stomach of pigs. Therefore HiPhos was active in the stomach and possibly also in the proximal small intestine (Pontoppidan *et al.*, 2012).

2.5 Conclusion

In conclusion, phytases from different microbial origins have different optimal temperatures, optimum pH, thermostability and proteolytic resistance. Therefore the efficiency of these phytases will differ under different environmental conditions. The amount of phytate in cereals and oilseeds differ between climatic conditions and therefore differ between countries. The phytate level in plants and the dietary composition of a diet also affects the results obtained for phytase trials. Trials discussed in the literature review were conducted in various countries with different commercial phytases supplemented to diets with different nutrient compositions, explaining the variation in results for several parameters.

Therefore it was important to conduct a study in South Africa, evaluating the effect of commercial phytases on production parameters and carcass characteristics under the same experimental conditions (ingredient and nutrient composition, dietary phytate levels and pelleting temperatures) to eliminate the effects external factors may have on the results.

2.6 References

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Chapter 3

Effect of phytase supplementation on production parameters and water intake of broiler chickens

Abstract

In several research publications, phytase supplementation was shown to liberate phytate bound phosphorus and nutrients, resulting in an improvement in production. However, these improvements differ depending on the level and type of commercial phytase used. A 32 day experiment evaluated the matrix values for a newly developed phytase (HiPhos) and the effect different commercial phytase enzymes had on the production parameters of broilers fed maize soya bean diets. A number of 5120 chickens were divided into 64 pens. Eight treatments with eight replicates were allocated to the pens in a randomized design. Treatments were: 1) Positive control (CON); 2) CON minus the matrix values for 1000 FYT HiPhos (NEG1000); 3) CON minus the matrix values for 1500 FYT HiPhos (NEG1500); 4) NEG1000 + 500 FTU Phyzyme (PHY); 5) NEG1000 + 1000 FYT HiPhos (HP1000); 6) NEG1000 + 500 FTU Natuphos (NATU); 7) NEG1000 + 1500 FYT Ronozyme (RZ); NEG1500 + 1500 FYT HiPhos (HP1500). Reducing the nutrients in the diets according to the matrix values of HiPhos phytase did not significantly influence body weight. Supplementation with 1500 FYT HiPhos phytase (HP1500) resulted in the highest body weight gain and this was the only treatment group that significantly differed from the Neg1500 treatment group. Therefore the matrix values for 1500 FYT HiPhos were verified. Feed and water intake were not affected by phytase supplementation. Significant differences for feed conversion ratio existed between the CON and NEG1500 group. Liveability was not affected by the treatments. An improvement in the European Production Efficiency Factor was noted in the positive control, HiPhos 1500 and Phyzyme treatments when compared to the NEG1500 group.

***keywords:** weight gain, live weight, feed intake, FCR, HiPhos matrix values

3.1. Introduction

Phosphorus (P) is an essential nutrient required for proper development and growth in all animals. It is needed for the maintenance and formation of the skeleton and plays a vital role in many metabolic processes (McDonald *et al.*, 2002; Suttle, 2010). Although not fully understood, P is also involved in appetite control (Bar & Hurwitz, 1984; Suttle, 2010) and food utilization efficiency (Suttle, 2010). Plants are good sources of P and the majority of broiler diets in South Africa are maize and soya bean based.

Generally cereals and oilseeds are rich in P, but approximately 85% and 62% of the P in maize and soya beans, respectively are in the form of phytate (Ravindran *et al.*, 1994). Phytate is the mixed cation salt of Myo-inositol-1,2,3,4,5,6-hexakis dihydrogen phosphate, also known as phytic acid (Reddy *et al.*, 1982). Phytate and phytic acid are naturally occurring compounds present in feedstuffs of plant origin (Reddy *et al.*, 1982) where it serves as the primary storage form of P (Hídvégi & Lásztity, 2002).

Unfortunately phytate bound P (phytate-P) is poorly utilised by monogastric animals (Nelson, 1967; Ballam *et al.*, 1984). Therefore supplementing monogastric diets with inorganic P is crucial in order to meet the dietary requirement for P. Inorganic P added to the diet not only increases feed and production costs, but a large proportion of phytate-P remains undigested and is excreted by the animal (Thacker *et al.*, 2003). In addition, high levels of undigested P in litter contribute to environmental pollution. Poultry litter is used as a fertilizer in soil, but excess P can leach into lakes and streams and may contribute to eutrophication (Nahm, 2007).

Phosphate groups in phytic acid are negatively charged and are capable of forming complexes with positively charged minerals (Davies & Olpin, 1979; Cheryan & Rackis, 1980; Lonnerdal *et al.*, 1989; Brink *et al.*, 1991), proteins (Hídvégi & Lásztity, 2002) and starch (Yoon *et al.*, 1983), rendering these nutrients unavailable for absorption. Phytate also elevates mucin excretion and therefore increases endogenous nitrogen and amino acid losses (Cowieson *et al.*, 2004). Furthermore, it is believed that phytate may have the ability to inhibit digestive enzymes such as α -amylase (Deshpande & Cheryan, 1984). However controversy in the literature exists regarding the inhibition of trypsin and pepsin (Singh & Krikorian, 1982; Deshpande & Damodaran, 1989; Vaintraub & Bulmaga, 1991; Caldwell, 1992). Therefore it is reasonable to assume that the hydrolysis of phytate may liberate phosphate groups, rendering it available for absorption. At the same time, hydrolysis may decrease the anti-nutritive effects of phytate and release phytate bound minerals, proteins and starch.

Phytase, which are naturally produced by plants and micro-organisms are the only enzymes known to release phosphate (PO_4) by means of phytate hydrolysis (Shaw *et al.*, 2010). Since 1991, exogenous microbial phytase enzymes have been commercially available for animal nutritional purposes. Supplementing poultry diets with phytase allows nutritionists to reduce the levels of available P, Ca, amino acids and metabolisable energy (ME) content in diet formulation. In order to reduce these nutrients in least cost feed formulations, nutritionists require the matrix values of the specific phytase enzyme used in the formulation. Matrix values indicate the estimated amount of extra nutrients (amino

acids, ME, P, Ca) available for absorption when phytase is added to the diet (Shelton *et al.*, 2004). The efficient releasing effect of any phytase on phytate bound amino acids and minerals and its effect on the energy value of the diet have to be quantified and verified; these processes are done by means of numerous feeding trials on widely differing diets in order to deduce matrix values.

In addition to increasing P availability, supplementation with phytase enzymes may improve poultry growth (Kornegay *et al.*, 1996; Zhang *et al.*, 1999; Johnston & Southern, 2000; Shirley & Edwards, 2003; Ravindran *et al.*, 2008; Shaw *et al.*, 2010; Aureli *et al.*, 2011; Pirgozliev *et al.*, 2011; Shaw *et al.*, 2011) and feed conversion ratio (FCR) (Shirley & Edwards, 2003; Ravindran *et al.*, 2008; Aureli *et al.*, 2011; Pirgozliev *et al.*, 2011; Shaw *et al.*, 2011). However, improvements may depend on the type and level of commercial phytase used and the dietary level of available P (aP) or non phytate phosphorus (npP). Production parameters (weight gain, feed intake, FCR) are important measurements of any dietary changes. Body weight has been shown to be a robust indicator of P levels (Kornegay *et al.*, 1996; Cabahug *et al.*, 1999; Viveros *et al.*, 2002; Rama Rao *et al.*, 2006; Panda *et al.*, 2007) and therefore body weight gain can be used as an indicator for assessing the efficacy of phytase enzymes.

As mentioned, phytase supplementation increases mineral availability and decreases the endogenous mineral excretion in chickens. Consequently, the osmolality within the gastrointestinal tract of the chicken may increase, which may result in an increased demand for water in order to maintain homeostasis (Cowieson *et al.*, 2004). Any dietary changes that may lead to an increment in water intake are expected to increase excreta and litter moisture, which in turn may affect the health and welfare of the chickens (Youssef *et al.*, 2011). A lack of information and published data on the effect that phytase has on water intake in broilers exists. Considering the negative effects wet litter may have on the health of the chick, it is important to determine the effects phytase may have on water intake.

Therefore the objectives of this study were to:

- i. Confirm the matrix values for 1000 FYT and 1500 FYT HiPhos, a newly developed phytase enzyme, using broiler growth as the response indicator
- ii. Determine if other commercial phytases have the ability to achieve the matrix values of HiPhos, using growth response as the response indicator
- iii. Evaluate the effect of each commercial phytase on the production parameters
- iv. Evaluate the effect of phytase supplementation on water intake
- v. Determine the production cost of broilers supplemented with the newly developed phytase (HiPhos) and compare it with broilers raised on the commercial diet without added phytase

3.2 Materials and Methods

3.2.1 Birds and housing

A total of 5120 day old broiler (Cobb 500) chicks were obtained from a commercial hatchery and transported for a distance of 50 km to the Mariendahl experimental farm of Stellenbosch University (Stellenbosch, Western Cape, South Africa). Eight treatments with eight replications per treatment were randomly allotted to 64 pens. At arrival, chicks were randomly allotted to one of eight treatment groups, with 80 birds assigned to each of 64 floor pens with a density of 21.8 chickens/m² (in accordance to the SAPA code of conduct). Each pen was equipped with two tube feeders, a bell drinker and an infrared lamp. Chickens were kept in two identical temperature controlled, positive pressure commercial type broiler houses. Environmental temperature and lightning within the houses was according to the Cobb 500 standard. The trial protocol was approved by the Animal Ethics Committee of Stellenbosch University, reference number: SU-ACUM12-00039.

Water and feed were supplied *ad libitum*. A starter diet (Table 3.3) was supplied at a rate of 586g per bird (over \pm 14 days), grower diet (Table 3.4) at a rate of 961g per bird (over \pm 7 days) and a finisher diet (Table 3.5) at a rate of 1289g per bird (over \pm 11 days). Feed consumption was recorded on a pen basis at weekly intervals until slaughter at 32 days of age. Individual feed intake was calculated as an average of the pens after correcting for mortality. To measure water intake, each pen was equipped with a 25L bucket connected to a bell drinker. The bucket was filled twice daily and water was weighed out each morning to determine daily water intake per pen. Water intake per bird was calculated as an average of the pen.

Body weight of all birds in a pen was measured at placement and weekly thereafter until slaughter at 32 days of age. Individual weights were calculated as an average of the pen weight after correction for mortality. Mortalities were recorded twice daily and all dead birds were weighed. Liveability is expressed as the percentage of birds surviving until slaughter expressed as a percentage of the total number placed. From these data feed conversion ratio (FCR) (Equation 1), European production efficiency factor (EPEF) (Equation 2) and the economics of the experimental diets (Equation 3, Equation 4, Equation 5) were calculated. The formulae used are shown below:

$$\text{Equation 1} \quad \text{FCR} = \frac{\text{Cumulative feed intake per chick (g)}}{\text{Average live weight gain per chick}}$$

$$\text{Equation 2} \quad \text{EPEF} = \frac{\text{Liveability (\%)} \times \text{Live weight gain (kg)}}{\text{Age (days)} \times \text{FCR}} \times 100$$

$$\text{Equation 3} \quad \text{Cost/kg weight gain/phase} = \frac{\text{Cost per kg diet (ZAR)} \times \text{Feed intake per chick per phase (kg)}}{\text{Live weight gain per chick per phase (kg)}}$$

$$\text{Equation 4} \quad \text{Total feed cost/chick} = \sum (\text{cost per kg diet per phase} \times \text{average intake during phase})$$

$$\text{Equation 5} \quad \text{Total feed cost (ZAR)/kg live weight gained} = \frac{\text{Total feed cost/chick}}{\text{Cumulative live weight gain}}$$

3.2.2 Treatments and experimental diets

The chicks were assigned to eight different treatment diets. The eight experimental treatments are described in Table 3.1. The main differences between the diets (treatments) were the reduction of nutrients according to the matrix values of HiPhos and the inclusion or not of different phytases. The positive control (CON) was a commercial diet with no added phytase. To formulate the negative controls (NEG1000 and NEG1500), the matrix values for 1000 FYT and 1500 FYT HiPhos were subtracted from the specifications of the positive control. The commercial phytases were supplemented to the negative controls to determine if they were sufficient in releasing the amount of minerals and nutrients specified by the matrix values. The different phytases evaluated were HiPhos (DSM Nutritional Products, Basel, Switzerland), Ronozyme (DSM Nutritional Products, Basel, Switzerland), Natuphos (BASF, Ludwigshafen, Germany) and Phyzyme (Danisco Animal nutrition, Copenhagen, Denmark). HiPhos, Ronozyme and Phyzyme are 6-phytases and Natuphos is a 3-phytase. Ronozyme, Natuphos and Phyzyme were derived from *Peniophora lycii*, *Aspergillus ficuum* and *Escherichia coli*, respectively, while mass produced in *Aspergillus oryzae*, *Aspergillus niger* and *Schizosaccharomyces pombe*, respectively. Whereas HiPhos is the first phytase derived from two synthetic genes, both mimicking a phytase gene from a *Citrobacter braakii* strain and mass produced in *Aspergillus oryzae*.

Diets were mixed at Mariendahl experimental farm, Stellenbosch. All diets were pelleted at 75 °C. Inclusion rates of each phytase enzyme in the respective diet were based on the manufacturer's recommended rates of addition. Therefore inclusion levels of all the phytases represents the standard dose and the P equivalence for the enzymes (excluding 1500 FYT HiPhos) are similar. Numerous studies have defined a unit of phytase activity (FTU or FYT) as the amount of enzyme that catalyses the release of 1 µMol inorganic orthophosphate per minute from 0.0051 mol/L sodium phytate at pH 5.5 and temperature of 37 °C (Engelen *et al.*, 1993). The phytase units declared do not always represent the amount of P released in the gastrointestinal tract of the animal because the pH and temperature conditions in the assays differ from the pH and temperature encountered in the different parts of the gastrointestinal tract of the animal.

Dietary samples of the all the diets were send to DSM (Biopract GmbH, Berlin, Germany) to determine phytase activity. Phytase activity in the diets was determined using the ISO 30024 (International Organization for standardization, 2009) procedure. The phytase units added to the diets and the analysed phytase activity in the grower and finisher diets are shown in Table 3.2. The nutrient and ingredient composition of all three maize soya bean basal diets for each phase are shown in Table 3.3, Table 3.4 and Table 3.5.

3.2.3 Statistical analysis

Analysis of variance was performed on pen means data using the general linear models (GLM) and ANOVA procedures of SAS (2009) with treatment as the main effect. All the parameters were tested for normality and homoscedasticity before analysis. Significance was declared at $P \leq 0.05$. Means was separated with a Bonferroni post hoc test (SAS, 2009). ADG was determined by means of fitting simpler linear regression of the weight over time. The slope of resulting regression function is ADG and was used to compare animals between treatments.

Table 3.1 A description of the dietary treatments used throughout the trial

Treatment	Description
CON	Normal specification of full feed with no added phytase
NEG1000	Normal specification of full feed minus matrix values of 1000 FYT HiPhos
NEG1500	Normal specification of full feed minus matrix values of 1500 FYT HiPhos
PHY	Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 500 FTU Phyzyme/kg diet
HP1000	Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 1000 FYT HiPhos/kg diet
NATU	Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 500 FTU Natuphos/kg diet
RZ	Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 1500 FYT Ronozyme/kg diet
HP1500	Normal specification of full feed minus matrix values of 1500 FYT HiPhos + 1500 FYT HiPhos/kg diet

Table 3.2 Phytase units (FTU) and analysed phytase activity rates in grower and finisher diets

Phytase	Declaration (FTU or FYT)	Analysed activity (U/kg) (Grower)	Analysed activity (U/kg) (Finisher)
Phyzyme	500	462	395
Natuphos	500	638	944
Ronozyme	1500	1525	1495
HiPhos	1000	710	1331
HiPhos	1500	1302	1399

Table 3.3 Ingredient and calculated nutrient composition of broiler starter diets used in the trial

	CON	NEG1000	NEG1500		
Ingredients (g/kg)					
Maize meal	512.46	573.64	561.09		
Fish meal	13.56	10.00	10.00		
Soya bean meal (48% CP) ¹	300.00	300.00	300.00		
Sunflower O/C (36% CP)	60.00	60.00	60.00		
Canola, pressed	40.00	31.70	24.85		
Wheaten Bran	10.98	0.48	21.21		
Ground limestone (80%)	9.92	9.29	9.13		
L-Lysine	0.57	0.73	0.76		
DL-Methionine	1.61	1.64	1.68		
Mono-calcium phosphate	13.32	4.88	3.65		
Salt	4.57	4.62	4.32		
Veg Acid Oil (Soil)	30.00	0.00	0.00		
Premix	3.00	3.00	3.00		
				Matrix values (g/kg)	
Calculated nutritional value (g/kg)				HP1000 ³	HP1500 ⁴
Dry matter	894.00	892.46	891.90	--	--
Moisture	103.00	107.54	108.10	--	--
Crude protein	219.00	217.05	216.75	1.95	2.25
Crude fibre	45.93	45.66	46.75	--	--
Calcium	9.00	7.22	6.92	1.78	2.08
Available phosphorus (aP)	4.32	2.57	2.34	1.71	1.98
Digestible phosphorus (dP)	4.11	2.61	2.41	1.5	1.7
Ca:aP	2.08	2.81	2.96	--	--
Ca:dP	2.19	2.77	2.87	--	--
AME ²	124.68	121.89	121.46	2.79	3.22
Digestible Lysine	11.00	10.92	10.91	0.08	0.09
Digestible Methionine	4.94	4.92	4.62	0.02	0.02
Digestible sulphur amino acids	8.06	8.02	8.01	0.04	0.05
Digestible Tryptophan	2.34	2.30	2.30	0.04	0.04
Digestible Isoleucine	8.97	8.90	8.85	0.07	0.12
Digestible Leucine	16.88	17.00	16.87	--	0.01
Digestible Threonine	7.66	7.58	7.53	0.08	0.13
Sodium	2.00	2.01	2.01	--	--
Potassium	9.07	9.05	9.19	--	--

CON: Normal specification of full feed

NEG1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos

NEG1500: Normal specification of full feed minus matrix values of 1500 FYT HiPhos

¹ Crude protein² AME: Apparent metabolisable energy³ HiPhos phytase (1000 FYT)⁴ HiPhos phytase (1500 FYT)

-- No value available

Table 3.4 Ingredient and calculated nutrient composition of broiler grower diets used in the trial

	CON	NEG1000	NEG1500		
Ingredients (g/kg)					
Maize meal	608.66	605.29	612.20		
Fish meal	20.71	0.00	0.00		
Soya bean meal (48% CP) ¹	316.20	284.46	282.81		
Sunflower O/C (36% CP)	0.00	70.00	70.00		
Ground limestone (80%)	10.82	10.57	10.31		
L-Lysine	0.00	0.98	0.99		
DL-Methionine	1.92	1.84	1.84		
Monocalcium phosphate	11.48	4.04	2.87		
Salt	4.21	4.55	4.55		
Veg Acid Oil (Soil)	23.00	15.26	11.43		
Premix	3.00	3.00	3.00		
				Matrix values (g/kg)	
Calculated nutritional value (g/kg)				HP1000 ³	HP1500 ⁴
Dry matter	888.35	892.06	891.53	--	--
Moisture	108.65	107.93	108.10	--	--
Crude protein	200.00	198.00	197.69	2.00	2.31
Crude fibre	34.03	43.67	43.77	--	--
Calcium	8.80	7.02	6.72	1.78	2.08
Available phosphorus (aP)	3.88	2.11	1.88	1.77	2.00
Digestible phosphorus (dP)	3.71	2.21	2.01	1.50	1.70
Ca:aP	22.68	33.27	35.74	--	--
Ca:dP	23.72	31.76	33.43	--	--
AME ²	129.70	126.90	126.50	0.66	0.77
Digestible Lysine	10.05	9.92	9.90	0.13	0.15
Digestible Methionine	4.84	4.75	4.74	0.09	0.10
Digestible sulphur amino acids	7.60	7.56	7.55	0.04	0.05
Digestible Tryptophan	2.11	2.09	2.08	0.02	0.03
Digestible Isoleucine	8.31	8.12	8.10	0.19	0.21
Digestible Leucine	16.26	15.74	15.74	0.52	0.52
Digestible Threonine	7.07	6.83	6.82	0.24	0.25
Sodium	1.90	1.90	1.90	--	--
Potassium	8.17	8.63	8.62	--	--

CON: Normal specification of full feed

NEG1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos

NEG1500: Normal specification of full feed minus matrix values of 1500 FYT HiPhos

¹ Crude protein² AME: Apparent metabolisable energy³ HiPhos phytase (1000 FYT)⁴ HiPhos phytase (1500 FYT)

-- No value available

Table 3.5 Ingredient and calculated nutrient composition of broiler finisher diets used in the trial

	CON	NEG1000	NEG1500		
Ingredients (g/kg)					
Maize meal	628.00	621.27	623.11		
Fish meal	29.08	0.00	0.00		
Soy bean meal (48% CP) ¹	271.95	261.10	251.48		
Sunflower O/C (36% CP)	0.00	59.15	70.00		
Canola, pressed	10.00	10.00	10.00		
Ground limestone (80%)	10.88	10.69	10.46		
L-Lysine	0.10	1.14	1.26		
DL-Methionine	1.74	1.76	1.73		
Monocalcium phosphate	11.20	4.34	3.16		
Salt	4.07	4.54	4.54		
Veg Acid Oil (Soil)	30.00	23.00	21.06		
Premix	3.00	3.00	3.00		
				Matrix values (g/kg)	
Calculated nutritional value (g/kg)				HP1000 ³	HP1500 ⁴
Dry matter	888.72	892.70	892.62	--	--
Moisture	108.28	107.30	107.38	--	--
Crude protein	190.00	187.97	187.66	2.03	2.34
Crude fibre	33.30	42.12	43.44	--	--
Calcium	8.80	7.02	6.72	1.78	2.08
Available phosphorus (aP)	3.96	2.13	1.91	1.83	2.05
Digestible phosphorus (dP)	3.71	2.21	2.01	1.5	1.7
Ca:aP	22.22	32.96	35.18	--	--
Ca:dP	23.72	31.76	33.43	--	--
AME ²	131.80	129.03	128.60	2.77	3.2
Digestible Lysine	9.60	9.52	9.50	2.77	3.2
Digestible Methionine	4.66	4.54	4.53	0.08	0.1
Digestible sulphur amino acids	7.92	7.25	7.24	0.12	0.13
Digestible Tryptophan	1.97	1.96	1.95	0.67	0.68
Digestible Isoleucine	7.80	7.65	8.85	0.01	0.02
Digestible Leucine	15.53	15.05	14.98	0.48	0.55
Digestible Threonine	6.71	6.46	6.44	0.25	0.27
Sodium	1.90	1.91	1.91	--	--
Potassium	7.44	8.13	8.11	--	--

CON: specification of full feed

NEG1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos

NEG1500: Normal specification of full feed minus matrix values of 1500 FYT HiPhos

¹ Crude protein

² AME: Apparent metabolisable energy

³ HiPhos phytase (1000 FYT)

⁴ HiPhos phytase (1500 FYT)

-- No value available

3.3 Results and Discussion

3.3.1 Live weight and weight gain

The effects that diets (CON, NEG1000 and NEG1500) and phytase supplementation had on growth are presented in Table 3.6 and Table 3.7. No differences ($P > 0.05$) in live weight were detected at hatch or on day 7. At day 14, live weight was significantly lower for chicks in the NEG1000, NEG1500 and RZ treatment groups compared to chicks in the HP1500 group. The other treatments were intermediary and statistically comparable.

On day 21, chickens in the CON treatment group had 5.8% heavier live weight compared to chickens in the Neg1500 treatment group. These two diets were formulated to have a 2.0 g/kg difference in available phosphorus (aP). A lack of significant difference ($P > 0.05$) existed between the CON treatment group and NEG1000 group. The NEG1000 diet was formulated to contain 1.8 g/kg less aP than the CON diet. In contrast with these findings, Shaw *et al.* (2010) reported a significant reduction in body weight for each 1.0 g/kg reduction in non phytate P (npP). The differences between the results in the current trial and the result reported by Shaw *et al.* (2010) might be partly explained by the differences in dietary Ca:P ratios between the studies.

Analysed Ca levels were higher than the calculated levels in the latter study (Shaw *et al.*, 2010). The analysed Ca:npP ratio in the study for the positive control (npP = 0.45, Ca = 1.37), marginal diet (npP = 0.35, Ca = 1.45) and negative control (npP = 0.25, Ca = 1.43), were 3.04:1; 4.14:1 and 5.72:1 respectively (Shaw *et al.*, 2010). Whereas the Ca:aP ratios in the current trial for the CON, NEG1000 and NEG1500 treatment in the starter diet was 2.08:1, 2.81:1 and 2.96:1 respectively. At wider ratios, Ca and P may form insoluble calcium phosphate in the gastrointestinal tract, reducing the absorption of these two minerals (Heaney & Nordin, 2002; Selle *et al.*, 2006). When the Ca levels in diets containing low aP levels increases, the Ca:aP ratio widens, resulting in decreased body weight gains (Rama Rao *et al.*, 2006). Furthermore, Ziaei *et al.* (2008) reported no significant differences in body weight gain in broilers when dietary Ca levels of 9.0 g/kg and aP levels of 4.5 g/kg in the diets were decreased to 6.0 g/kg and 3.4 g/kg respectively. The lack of agreement between these studies when evaluating the effect that aP content had on growth may be due to a number of factors including dietary Ca:aP ratio, dietary ingredients, variation in mineral source, vitamin D₃ level and processing (Ravindran *et al.*, 1995).

Results for body weight and weight gain on day 21 showed no differences ($P > 0.05$) between the CON, HP1000 and HP1500 treatment groups. In addition, the mean live weight of chickens receiving NEG1500 diets was significantly lower than chickens on the HP1000, HP1500 and CON diets. Compared to the NEG1500 group, supplementing chickens with 1000 FYT HiPhos (HP1000) or 1500 FYT HiPhos (HP1500) significantly increased live weight gain with 4.83% and 6.05%, respectively. However, these results were not as pronounced as found by Shaw *et al.* (2011) who reported that the addition of 1000 FYT or 2000 FYT HiPhos phytase to broiler diets (2.2 g/kg npP) increased body weight of 21 day old broilers with 15.5% and 17.1%, respectively.

Table 3.6 Mean (\pm standard deviation) live weights (g) of broilers grown from hatch up to day 32 receiving different dietary phytase treatments

Treatment	Day 0	Day 7	Day 14	Day 21	Day 28	Day 32
CON	44 \pm 0.6	176 \pm 5.6	460 ^{ab} \pm 11.0	955 ^b \pm 37.27	1523 ^{ab} \pm 39.1	1849 ^{ab} \pm 40.6
NEG1000	44 \pm 1.1	173 \pm 3.5	449 ^a \pm 8.3	918 ^{ab} \pm 19.47	1485 ^{ab} \pm 32.8	1817 ^{ab} \pm 48.4
NEG1500	44 \pm 0.5	167 \pm 6.6	446 ^a \pm 16.0	900 ^a \pm 12.27	1477 ^a \pm 38.6	1808 ^a \pm 48.2
PHY	44 \pm 0.8	171 \pm 6.2	460 ^{ab} \pm 8.8	928 ^{ab} \pm 28.43	1507 ^{ab} \pm 24.6	1849 ^{ab} \pm 32.1
HP1000	44 \pm 0.8	174 \pm 7.9	460 ^{ab} \pm 12.4	946 ^b \pm 25.27	1523 ^{ab} \pm 30.4	1867 ^{ab} \pm 30.6
NATU	44 \pm 0.8	175 \pm 4.1	461 ^{ab} \pm 10.6	941 ^{ab} \pm 30.30	1524 ^{ab} \pm 32.3	1873 ^{ab} \pm 43.0
RZ	44 \pm 0.6	171 \pm 4.5	448 ^a \pm 8.6	926 ^{ab} \pm 28.15	1502 ^{ab} \pm 22.7	1842 ^{ab} \pm 29.4
HP1500	44 \pm 0.6	177 \pm 3.7	471 ^b \pm 8.9	958 ^b \pm 21.74	1539 ^b \pm 25.5	1879 ^b \pm 39.9
P Value	0.774	0.122	0.001	0.001	0.004	0.008

^{a,b} Means within columns with different superscripts differ significantly ($P < 0.05$)

CON: Normal specification of full feed

NEG1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos

NEG1500: Normal specification of full feed minus matrix values of 1500 FYT HiPhos

PHY: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 500 FTU Phyzyme phytase

HP1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 1000 FYT HiPhos phytase

NATU: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 500 FTU Natuphos phytase

RZ: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 1500 FYT Ronozyme phytase

HP1500: Normal specification of full feed minus matrix values of 1500 FYT HiPhos + 1500 FYT HiPhos phytase

On day 28 and 32, significant differences in live weight and weight gain were noted between the HP1500 group and NEG1500 group. In addition, live weight of the HiPhos treatment group was heavier, but not significantly different from the CON after the grower and finishing phases. These results are indicative that the matrix values for 1500 FYT HiPhos are correct when using live weight as the response criteria. All the other treatment groups exhibited results intermediary and similar to the HP1500 and NEG1500 treatment groups. Similarly, Shaw *et al.* (2011) reported that Phyzyme and Ronozyme have the ability to replace 1.0 g/kg dietary npP levels when live weight was used as the response criteria, but these phytases were unable to replace 2.0 g/kg npP at day 21. However, Ronozyme was able to replace 2.0 g/kg npP at day 28 in the latter study (Shaw *et al.* 2011). In the same way, Viveros *et al.* (2002) supplemented broiler diets (npP = 3.5 g/kg) with 500 FTU Natuphos. After three weeks, the body weight, feed intake and FCR of chickens supplemented with phytase did not differ ($P > 0.05$) from chickens receiving diets with high (4.45 g/kg) npP levels.

Table 3.7 Mean (\pm standard deviation) cumulative live weight gains (g) of broilers grown from hatch up to day 32 receiving different dietary phytase treatments

Treatment	Day 0-7	Day 0-14	Day 0-21	Day 0-28	Day 0-32
CON	132 \pm 5.6	416 ^{ab} \pm 10.9	911 ^b \pm 36.9	1479 ^{ab} \pm 39.1	1805 ^{ab} \pm 40.7
NEG1000	128 \pm 3.9	405 ^a \pm 8.2	873 ^{ab} \pm 19.7	1441 ^{ab} \pm 32.8	1773 ^{ab} \pm 48.1
NEG1500	126 \pm 6.9	402 ^a \pm 16.3	856 ^a \pm 12.6	1433 ^a \pm 38.8	1764 ^a \pm 48.3
PHY	127 \pm 6.1	416 ^{ab} \pm 8.4	884 ^{ab} \pm 28.3	1463 ^{ab} \pm 24.4	1805 ^{ab} \pm 32.4
HP1000	130 \pm 7.9	416 ^{ab} \pm 12.1	901 ^b \pm 24.8	1479 ^{ab} \pm 30.2	1823 ^{ab} \pm 30.9
NATU	131 \pm 4.3	418 ^{ab} \pm 10.9	897 ^{ab} \pm 30.2	1480 ^{ab} \pm 32.6	1829 ^{ab} \pm 43.1
RZ	127 \pm 4.6	405 ^a \pm 8.7	882 ^{ab} \pm 28.4	1458 ^{ab} \pm 22.7	1798 ^{ab} \pm 29.4
HP1500	133 \pm 3.9	427 ^b \pm 8.7	914 ^b \pm 21.8	1495 ^b \pm 25.5	1834 ^b \pm 39.9
P value	0.147	<0.001	0.001	0.004	0.008

^{a,b} Means within columns with different superscripts differ significantly ($P < 0.05$)

CON: Normal specification of full feed

NEG1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos

NEG1500: Normal specification of full feed minus matrix values of 1500 FYT HiPhos

PHY: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 500 FTU Phyzyme phytase

HP1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 1000 FYT HiPhos phytase

NATU: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 500 FTU Natuphos phytase

RZ: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 1500 FYT Ronozyme phytase

HP1500: Normal specification of full feed minus matrix values of 1500 FYT HiPhos + 1500 FYT HiPhos phytase

The lack of significant difference observed between the CON and NEG1000 or CON and NEG1500 treatment groups with regards to live weight on the day of slaughter was unexpected. One possible explanation is an observed asymptomatic infection with *Escherichia coli* throughout all the treatment groups in the trial. High mortality rates (average of 8.13% for the flock) were observed in the trial due to *E. coli* infection. Not knowing the pathogen load on the surviving chickens, it is possible that the pathogen load could have acted as a confounding factor in this study and possibly influenced the outcome of the trial. Notwithstanding this possibility, chickens did exhibit body weight values higher than expected for this chicken line (Cobb, 2012). According to the nutrient specifications for Cobb 500, minimum dietary Levels of aP should be 4.5, 4.2 and 3.8 g/kg for starter, grower and finisher rations, respectively (Cobb, 2012). The NEG1500 starter, grower and finisher diet had 1.9 and 2.0 and 1.7 g/kg lower aP levels in comparison with the nutrient recommendations for the strain. However, despite the *E. coli* infection and these reductions in aP, chickens fed CON, NEG1000 and NEG1500 diets had body weight values (Table 3.7) higher than the expected values (1.78 kg) for the strain on day 32 (Cobb, 2012). Average daily gain (Table 3.10) was determined by means of fitting a simple linear regression of the weight over time with the slope of this curve representing the ADG. Average daily gain of broilers in the NEG1500 treatment was significantly lower than that of the CON, HP1000, NATU and HP1500 treatments while the HP1500 treatment was significantly higher than the NEG1500 and NEG1000 treatments. The other treatments were intermediary and statistically similar.

3.3.2 Feed intake

Loss of appetite can be an early characteristic of phosphorus deprivation (Suttle, 2010) but a loss of appetite was not observed in chickens fed diets low in aP in the current trial. Significant differences for cumulative feed intake were only observed during the starter phase with the NEG1500 treatment group consuming less feed than the PHY treatment group. Phosphorus levels and phytase supplementation did not affect feed intake in the grower or finisher phase. Similarly, Shaw *et al* (2011) reported a lack of significant difference for feed intake between broilers fed diets with different npP levels and HiPhos supplementation. Whereas Viveros *et al.* (2002) reported a significant difference in feed intake for broilers on diets with different npP levels, but Natuphos phytase supplementation did not increase feed consumption. On the contrary, Shaw *et al.* (2010) and Aureli *et al.* (2011) reported significant differences in feed intake between broilers on diets with different npP levels and in broilers receiving diets with phytase supplementation. However, dietary npP levels (0.8 g/kg) in the study of Aureli *et al.* (2011) were extremely low; moreover, Wu *et al.* (2006) noted a significant interaction between dietary npP levels and phytase on feed intake. Phytase increased feed intake in layers fed diets with 1.1 g/kg npP, but phytase supplementation to diets with 2.2 g/kg npP had no effect on intake. Therefore, differences in feed intake results between studies may be partially explained by dietary aP levels.

Table 3.8 Mean (\pm standard deviation) cumulative feed intake (g) with standard deviations of broilers grown from hatch up to day 32 receiving different dietary phytase treatments

Treatment	Day 0-7	Day 0-14	Day 0-21	Day 0-28	Day 0-32
CON	166 \pm 5.5	599 ^{ab} \pm 11.8	1347 \pm 30.9	2299 \pm 51.0	2904 \pm 36.1
NEG1000	165 \pm 4.7	602 ^{ab} \pm 9.6	1340 \pm 32.0	2293 \pm 55.5	2921 \pm 73.9
NEG1500	160 \pm 5.8	590 ^a \pm 13.2	1331 \pm 21.8	2305 \pm 53.6	2943 \pm 71.2
PHY	168 \pm 6.4	611 ^b \pm 9.9	1366 \pm 30.0	2343 \pm 28.7	2980 \pm 51.1
HP1000	163 \pm 7.5	603 ^{ab} \pm 9.9	1360 \pm 31.4	2317 \pm 43.7	2944 \pm 38.0
NATU	162 \pm 5.8	606 ^{ab} \pm 16.8	1367 \pm 39.1	2352 \pm 61.9	2986 \pm 76.8
RZ	170 \pm 7.7	608 ^{ab} \pm 10.3	1359 \pm 27.3	2322 \pm 55.7	2954 \pm 71.3
HP1500	167 \pm 5.6	608 ^{ab} \pm 5.2	1370 \pm 22.5	2358 \pm 65.3	2983 \pm 83.0
P-value	0.050	0.016	0.116	0.125	0.160

^{a,b} Means within columns with different superscripts differ significantly ($P < 0.05$)

CON: Normal specification of full feed

NEG1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos

NEG1500: Normal specification of full feed minus matrix values of 1500 FYT HiPhos

PHY: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 500 FTU Phyzyme phytase

HP1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 1000 FYT HiPhos phytase

NATU: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 500 FTU Natuphos phytase

RZ: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 1500 FYT Ronozyme phytase

HP1500: Normal specification of full feed minus matrix values of 1500 FYT HiPhos + 1500 FYT HiPhos phytase

3.3.3 Feed conversion ratio

The effect of phytase supplementation on weekly cumulative FCR is presented in Table 3.9. From day 21 until the end of the trial the CON treatment group had the best FCR. In week 3, the CON treatment group had significantly better cumulative FCR than the PHY, RZ and NEG1500 treatments groups, but significant differences were only observed between the CON and NEG1500 treatment groups during week 4. Final FCR (0-32 days) indicated the CON and HP1000 treatments to be lower ($P < 0.05$) than the NEG1500 treatment group. All other treatments were intermediary and statistically similar.

Controversy in literature exists regarding the effect of phytase supplementation on FCR in broilers. Some studies reported an improvement in feed conversion ratio (Shirley & Edwards, 2003; Ravindran *et al.*, 2008; Aureli *et al.*, 2011; Pirgozliev *et al.*, 2011; Shaw *et al.*, 2011) whereas others found no significant improvements when phytase was added to diets containing low npP levels (Zhang *et al.*, 1999; Johnston & Southern, 2000; Shaw *et al.*, 2010). However, in the case of Shirley & Edwards (2003), Aureli *et al.* (2011) Pirgozliev *et al.* (2011) and Shaw *et al.* (2011), significant improvement of the FCR was dependent on the phytase levels.

Table 3.9 Mean (\pm standard deviation) cumulative feed conversion ratios of broilers grown from hatch up to day 32 receiving different dietary phytase treatments

Treatment	Day 0-7	Day 0-14	Day 0-21	Day 0-28	Day 0-32
CON	1.26 ^{ab} \pm 0.06	1.44 ^{ab} \pm 0.03	1.48 ^a \pm 0.04	1.56 ^a \pm 0.02	1.61 ^a \pm 0.03
NEG1000	1.29 ^{ab} \pm 0.05	1.49 ^{bc} \pm 0.04	1.53 ^{ab} \pm 0.03	1.59 ^{ab} \pm 0.02	1.65 ^{ab} \pm 0.02
NEG1500	1.27 ^{ab} \pm 0.05	1.47 ^{abc} \pm 0.05	1.56 ^b \pm 0.03	1.61 ^b \pm 0.04	1.67 ^b \pm 0.03
PHY	1.33 ^b \pm 0.10	1.47 ^{abc} \pm 0.03	1.55 ^b \pm 0.03	1.60 ^{ab} \pm 0.02	1.65 ^{ab} \pm 0.03
HP1000	1.26 ^{ab} \pm 0.03	1.45 ^{abc} \pm 0.03	1.51 ^{ab} \pm 0.03	1.57 ^{ab} \pm 0.02	1.61 ^a \pm 0.03
NATU	1.24 ^a \pm 0.06	1.45 ^{abc} \pm 0.05	1.52 ^{ab} \pm 0.02	1.59 ^{ab} \pm 0.04	1.63 ^{ab} \pm 0.02
RZ	1.34 ^b \pm 0.05	1.50 ^c \pm 0.05	1.54 ^b \pm 0.06	1.59 ^{ab} \pm 0.04	1.64 ^{ab} \pm 0.04
HP1500	1.25 ^{ab} \pm 0.04	1.42 ^a \pm 0.02	1.50 ^{ab} \pm 0.04	1.58 ^{ab} \pm 0.02	1.63 ^{ab} \pm 0.02
P-value	0.009	0.002	0.001	0.008	0.004

^{a,b} Means within columns with different superscripts differ significantly ($P < 0.05$)

CON: Normal specification of full feed

NEG1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos

NEG1500: Normal specification of full feed minus matrix values of 1500 FYT HiPhos

PHY: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 500 FTU Phyzyme phytase

HP1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 1000 FYT HiPhos phytase

NATU: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 500 FTU Natuphos phytase

RZ: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 1500 FYT Ronozyme phytase

HP1500: Normal specification of full feed minus matrix values of 1500 FYT HiPhos + 1500 FYT HiPhos phytase

3.3.4 European Production Efficiency Factor (EPEF) and liveability

Results for EPEF and liveability are shown in Table 3.10. Liveability is expressed as the percentage of birds surviving until slaughter. No significant differences were observed for liveability between treatment groups (Table 3.10). These findings are in agreement with results of Yan *et al.* (2003). In contrast, Waldroup *et al.* (2000) reported npP levels to have an effect on mortality, however, an increase in mortality only appeared when npP levels was lower than 2.5 g/kg. In the current study npP levels of the diets were not available, but the lowest aP levels for a treatment diet was 1.88 g/kg. However the decrease in aP did not significantly influence liveability or mortality percentage compared to the CON treatment group (aP = 3.88 g/kg). The liveability for the flock was lower than expected and this was a result of the *E. coli* infection in the flock.

The EPEF of the NEG1500 treatment group was significantly lower ($P < 0.05$) than that of the CON and HP1500 treatments, with the CON treatment being higher ($P < 0.05$) than the NEG1500 treatment but not different ($P > 0.05$) from the HP1500 treatment. None of the other treatments showed differences ($P > 0.05$) between the CON or NEG1500 treatment groups. The EPEF takes liveability into account and therefore the low liveability due to the *E.coli* infection in the flock decreased the EPEF in the treatment groups. As mentioned before, there were no significant differences for liveability between the treatment groups, but the NEG1000 group had a tendency for a higher liveability. The higher liveability in the NEG1000 group may have increased the EPEF of the group and contributed to the lack of significant differences for EPEF between the broilers in the NEG1000 treatment group and broilers supplemented with phytase.

Table 3.10 Mean (\pm standard deviation) European production efficiency factor (EPEF), liveability (%) and average daily gain (ADG) of broilers grown from hatch up to day 32 receiving different dietary phytase treatments

Treatment	EPEF	Liveability (%)	ADG (g)
CON	332.93 ^c \pm 10.34	95 \pm 3.0	58.46 ^{bc} \pm 1.36
NEG1000	318.26 ^{abc} \pm 9.74	95 \pm 1.8	57.40 ^{ab} \pm 1.42
NEG1500	300.18 ^a \pm 17.74	91 \pm 4.4	56.96 ^a \pm 1.42
PHY	309.83 ^{bc} \pm 16.03	91 \pm 4.0	57.92 ^{abc} \pm 1.04
HP1000	321.49 ^{abc} \pm 10.12	91 \pm 3.9	58.33 ^{bc} \pm 1.73
NATU	317.06 ^{abc} \pm 19.54	91 \pm 4.0	58.40 ^{bc} \pm 1.32
RZ	311.30 ^{abc} \pm 10.57	91 \pm 2.6	57.89 ^{abc} \pm 1.05
HP1500	326.13 ^{bc} \pm 14.74	90 \pm 6.8	58.94 ^c \pm 1.24
P-value	0.001	0.098	<0.001

^{a,b} Means within columns with different superscripts differ significantly ($P < 0.05$)

CON: Normal specification of full feed

NEG1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos

NEG1500: Normal specification of full feed minus matrix values of 1500 FYT HiPhos

PHY: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 500 FTU Phyzyme phytase

HP1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 1000 FYT HiPhos phytase

3.3.5 Production costs for experimental diets

The cost of one kg live weight gained (excluding the cost of day old chick) was the least for chickens fed on the HP1500 diets in the starter and grower period (Table 3.11). During the finisher period, the HP1000 treatment group had the least cost/kg live weight gain. The prices of the Natuphos, Phyzyme and Ronozyme phytase were not available and therefore production costs of broilers supplemented with these phytases could not be determined.

Table 3.11 Production cost per phase for experimental broiler diets supplemented with HiPhos Phytase

Treatment	Starter			Grower			Finisher		
	CON	HP 1000	HP 1500	CON	HP 1000	HP 1500	CON	HP 1000	HP 1500
Cost / kg diet (ZAR)	2.86	2.74	2.72	2.85	2.67	2.66	2.85	2.66	2.64
Feed cost / kg live weight gained	4.12	3.97	3.88	4.31	4.17	4.15	4.97	4.57	4.63

CON: Normal specification of full feed

HP1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 1000 FYT HiPhos phytase

HP1500: Normal specification of full feed minus matrix values of 1500 FYT HiPhos 1500+ 1500 FYT HiPhos phytase

ZAR: South African Rand

The total feed cost/chicken produced (Table 3.12) was the lowest for chickens in the HP1000 treatment group. Even though cost/kg diet was less for the HP1500 diet compared to the HP1000 diet, the cumulative feed conversion ratio of HP1500 chickens was higher, and therefore chickens consumed more feed to produce 1 kg live weight. At the end of the production cycle, the costs to produce 1 kg chicken when broilers were fed diets supplemented with 1000 FYT HiPhos (HP1000) were R0.27 less compared to broilers fed the commercial diet without phytase (CON).

Table 3.12 Total production cost for broilers receiving the basal diets or diets supplemented with HiPhos phytase

	CON	HP1000	HP1500
Total feed cost / chick (ZAR)	8.29	7.88	7.94
Total feed cost (ZAR) / kg live weight gained	4.59	4.32	4.33

3. CON: Normal specification of full feed

HP1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 1000 FYT HiPhos phytase

HP1500: Normal specification of full feed minus matrix values of 1500 FYT HiPhos + 1500 FYT HiPhos phytase

ZAR: South African Rand

3.3.6 Water intake

Cowieson *et al.* (2006) suggested that increased liberation of Na and K by means of phytase, together with the possibility that phytase may decrease endogenous Na and Ca excretion (Cowieson *et al.*, 2004) may increase the osmolality in the gastrointestinal tract. The increased osmolality may increase the demand for water to maintain homeostasis. In the current trial, differences ($P < 0.05$) for weekly cumulative water intake (Table 3.13) were only observed during week one between the positive and two negative controls.

Table 3.13 Mean (\pm standard deviation) cumulative water intake (L) of broilers grown from hatch up to day 32 receiving different dietary phytase treatments

Treatment	Day 0 - 7	Day 0 - 14	Day 0 - 21	Day 0 - 28	Day 0 - 32
CON	0.371 ^a \pm 0.01	1.254 \pm 0.04	2.837 \pm 0.09	4.680 \pm 0.13	6.086 \pm 0.23
NEG1000	0.399 ^b \pm 0.01	1.290 \pm 0.03	2.592 \pm 0.62	4.777 \pm 0.12	6.096 \pm 0.09
NEG1500	0.390 ^b \pm 0.01	1.277 \pm 0.03	2.815 \pm 0.10	4.735 \pm 0.11	6.150 \pm 0.15
PHY	0.384 ^{ab} \pm 0.01	1.304 \pm 0.03	2.870 \pm 0.04	4.770 \pm 0.12	6.202 \pm 0.21
HP1000	0.382 ^{ab} \pm 0.01	1.291 \pm 0.03	2.865 \pm 0.09	4.787 \pm 0.16	6.244 \pm 0.31
NATU	0.380 ^{ab} \pm 0.01	1.276 \pm 0.04	2.829 \pm 0.08	4.699 \pm 0.12	6.162 \pm 0.16
RZ	0.384 ^{ab} \pm 0.01	1.285 \pm 0.02	2.880 \pm 0.06	4.815 \pm 0.10	6.299 \pm 0.16
HP1500	0.385 ^{ab} \pm 0.02	1.306 \pm 0.03	2.897 \pm 0.13	4.808 \pm 0.19	6.254 \pm 0.19
P-value	0.002	0.059	0.068	0.554	0.653

^{a,b} Means within columns with different superscripts differ significantly ($P < 0.05$)

CON: Normal specification of full feed

NEG1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos

NEG1500: Normal specification of full feed minus matrix values of 1500 FYT HiPhos

PHY: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 500 FTU Phyzyme phytase

HP1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 1000 FYT HiPhos phytase

NATU: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 500 FTU Natuphos phytase

RZ: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 1500 FYT Ronozyme phytase

HP1500: Normal specification of full feed minus matrix values of 1500 FYT HiPhos + 1500 FYT HiPhos phytase

Phytase supplementation did not significantly influenced water intake during the trial. In contrast, Debicki-Garnier & Hruby (2003) noted a variation in water intake for poultry supplemented with phytase. When phytase was added in barley-based diets, Juanpere *et al.* (2004) noted a higher water intake in phytase supplemented broilers when compared to the negative control group but similar water intake compared to the positive control. There was however no difference in water:feed ratio between chickens in the positive control, negative control or phytase treatment groups and therefore water intake only increased with an increase in feed intake and live weight (Juanpere *et al.*, 2004). To the best of our knowledge, published research regarding the effects phytase has on water intake in broilers are scarce. Shaw *et al.* (2006) illustrated that elevated levels of Ca, P, Na, CL and K did not increase water intake in growing pigs, but faecal water excretion tended to increase. Phytase supplementation did not increase water intake in studies done on sows (Kemmer *et al.*, 1997) and horses (Van Doorn *et al.*, 2004).

3.4 Conclusion

Reducing dietary aP, Ca and nutrients levels according to the matrix values of 1500 FYT HiPhos only decreased weekly live weight of broilers during one period (14 to 21 days). Broilers in the CON and HP1500 groups had significantly higher ADGs compared to broilers in the NEG1500 treatment group. Furthermore, supplementing the NEG1500 diet with 1500 FYT HiPhos significantly increased live weight from day 14 up to day 32. Therefore, if considering only growth response as the response criteria for testing the matrix values of the HiPhos phytase, the matrix values for 1500 FYT HiPhos can be considered accurate and can be used in formulating broiler diets supplemented with 1500 FYT HiPhos. Feeding broilers diets with reduced dietary aP, Ca and nutrient levels according to the matrix values of 1000 FYT HiPhos (HP1000) did not significantly decrease growth parameters during the trial. Therefore, even though growth parameters of chickens supplemented with 1000 FYT HiPhos (HP1000) did not significantly differ from chickens in the positive control group (CON), the matrix values cannot be confirmed. An asymptomatic infection with *E. coli* was observed in the trial. Therefore the possibility exist that the pathogen load in the surviving chickens acted as a confounding factor, thus influencing the results in the trial. Furthermore, various authors reported a reduced final body weight when aP levels in broiler diets decreased. Therefore it cannot be accepted that feeding diets with the nutrient specification used in the NEG1500 treatment to commercial broilers will be sufficient in maintaining standard growth rates, even though this was the outcome in the current trial. Live weight gains did not differ between the broilers supplemented with 1000 FYT HiPhos, 500 FTU Natuphos, 500 FTU Phyzyme or 1500 FYT Ronozyme. Therefore the conclusion can be made that these phytases are equally effective in hydrolysing phytate.

Phytase supplementation had no significant effect on cumulative feed or water intake at the end of the trial. Cumulative FCR at the end of the trial was significantly lower for chickens in the CON and HP1000 group compared to the NEG1500 treatment group. The EPEF of chickens in the CON and HP1500 treatment group was significantly higher than chickens in the NEG1500 group. What is more, results from the current trial indicates that supplementing standard broiler diets with HiPhos phytase is more economically viable than feeding broilers diets without phytases.

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Chapter 4

Influence of phytase enzymes on carcass characteristics and skeletal parameters of broilers

Abstract

A 32 day experiment was conducted to verify the matrix values of a newly developed phytase (HiPhos) and to evaluate the effect of different commercial phytase enzymes on bone mineralisation and carcass characteristics of broilers. Five thousand one hundred and twenty chicks were divided into 64 pens. Eight treatments with eight replicates were randomly allocated to the pens in two identical broiler houses. Treatments were: 1) Positive control (CON); 2) CON minus the matrix values for 1000 FYT HiPhos (NEG1000); 3) CON minus the matrix values for 1500 FYT HiPhos (NEG1500); 4) NEG1000 + 500 FTU Phyzyme (PHY); 5) NEG1000 + 1000 FYT HiPhos (HP1000); 6) NEG1000 + 500 FTU Natuphos (NATU); 7) NEG1000 + 1500 FYT Ronozyme (RZ); NEG1500 + 1500 FYT HiPhos (HP1500). At 29 days of age, one bird per pen (eight birds per treatment) was slaughtered for tibia collection to evaluate bone status. Tibia breaking strength (N) of the CON treatment group was significantly higher than the NEG1000 and NEG1500 treatment group. Supplementation with 1000 FYT HiPhos or 500 FTU Natuphos significantly increased tibia breaking strength (N) compared to the NEG1000 treatment group. Fat free dry bone weight of the NATU and HP1000 treatment group were significantly higher than the NEG1000 treatment group and similar to the CON treatment group. Bone ash content of the CON treatment group was significantly higher than the NEG1000 and NEG1500 treatment groups. All the other treatment groups were intermediate to the negative controls and positive control. Treatment did not have an effect on Ca% and P% of the tibia. On day 33, one bird per pen was slaughtered to evaluate carcass characteristics. No treatment differences were found regarding dressing percentage, portion sizes, CIE-Lab colour for the breast or pH for the breast and thigh. The CIE L* value for the thighs from chickens in the HP1500 group was significantly higher, and therefore appeared lighter, compared to the NATU and RZ treatment groups.

***keywords:** bone breaking strength, muscle pH, tibia, ash, CIE-Lab, dressing %

4.1 Introduction

Plants are a major source of dietary phosphorus (P), but approximately 60 to 85% of the total P in common feed ingredients is bound in the form of phytic acid or phytate (Ravindran *et al.*, 1994; Selle & Ravindran, 2007). Phosphate groups in phytic acid are negatively charged and are capable of forming complexes with positively charged minerals (Davies & Olpin, 1979; Cheryan & Rackis, 1980; Lonnerdal *et al.*, 1989; Brink *et al.*, 1991), proteins (Hídvégi & Lásztity, 2002) and starch (Yoon *et al.*, 1983), rendering these nutrients unavailable for absorption.

Phytate bound P is poorly utilised by monogastric animals and therefore P should be added to the diet in the form of feed phosphates to meet the P requirements of monogastric animals. Phytase has the ability to dephosphorylate phytate; releasing phytate bound nutrients and phosphorus. Consequently, dietary supplementation with microbial phytase allows reduction of this mineral during diet formulation.

Phosphorus is an important nutrient required for proper development and growth in all animals (McDonald *et al.*, 2002). Together with calcium (Ca) and magnesium (Mg), it forms the structural components of the skeleton (Pond *et al.*, 2005). Before the formation of hydroxyapatite crystals for skeletal ossification occurs, the product of Ca and P ions in the fluid surrounding the bone matrix should exceed a critical minimum level (Pond *et al.*, 2005). Bone status is very important in poultry production and can be used as an indicator of mineral adequacy of the diet.

The degree of bone mineralisation affects bone strength. Phosphorus and/or Ca deficiency can increase bone breakage and defects in the bone (Brenes *et al.*, 2003). This defects or breakage of the tibia and femur during processing results in downgrading of the meat. Furthermore, fracturing of the clavicle bones can cause bloody breast meat or bloodiness on the *Pectoralis minor* muscle (Driver *et al.*, 2006). Along with the downgrading of meat, mineralisation defects are associated with an increased risk of bone fractures as well as deformity of the metatarsi. As a result, these fractures and deformities influences animal welfare and evidently affect feed intake and production (Orban *et al.*, 1999). The release and bioavailability of phytate bound P (phytate-P) through the use phytase can be evaluated by responses in live weight gain and bone development (Orban *et al.*, 1999). Live weight performance and mineral retention are good indicators of dietary change, but bone mineral concentrations are generally better indicators of P status and are more accurate in determining P bioavailability of the diet (Brenes *et al.*, 2003). In addition to bone ash, bone breaking strength and bone weight can be used to evaluate bone mineralisation in poultry (Onyango *et al.*, 2003).

As mentioned above, phytate is capable of forming complexes with other nutrients. In addition, phytate increases endogenous amino acid losses (Cowieson *et al.*, 2004) and it is also believed to inhibit digestive enzymes (Singh & Krikorian, 1982; Deshpande & Damodaran, 1989; Maenz, 2001). Therefore phytase supplementation should increase the availability of protein and starch. A shift in available energy or protein may change the rate of protein and fat deposition (Kies *et al.*, 2005) and as a result have an effect on certain carcass characteristics, for example dressing percentage and cut yield.

It is also important to consider the effects that phytase may have on the appearance and physical characteristics of the meat. These factors determine the quality of the meat and are determined by the consumer. Meat colour and pH of the meat are critical attributes to meat quality. The visual appearance of meat is the primary and most important factor determining retail selection (Barbut, 2001). The colour of the meat is of critical importance, because consumers often reject products if the colour varies from the expected normal appearances (Qiao *et al.*, 2001). At the same time, a well-known relationship between meat colour and meat pH exists (Mancini & Hunt, 2005). The Lightness (L^* values) of raw breast fillets is negatively correlated to the pH of the meat (Qiao *et al.*, 2001). Furthermore, the rate of pH decline and the temperature of the muscle when pH_u is attained play a role in the water-holding capacity, texture and tenderness of the meat (Richardson & Mead, 1999). It is also important to look at the effect feed additives may have on portion sizes, because the wholesale prices per kilogram differs (SAPA, 2013) between portions and this may affect profits if chickens are sold as commercial cuts. Phytase has shown to have no negative effects on meat colour, muscle pH (Han *et al.* 2009) or portion sizes (Scheideler & Ferket, 2000). However literature regarding the changes in carcass characteristics due to phytase supplementation is limited and more research is warranted to quantify the effects phytase supplementation may have on carcass characteristics.

The objectives of this study was to:

- i) Confirm the matrix values of HiPhos, using bone parameters (bone weight, tibia strength, ash, mineral content) as a response criteria
- ii) Determine if other commercial phytases have the ability to achieve the matrix values of HiPhos, using bone parameters as a response criteria
- iii) Determine if phytase has an effect on carcass characteristics (muscle pH, meat colour, carcass component yield, dressing percentage)

4.2 Materials and Methods

Details on the experimental outlay, animals, diets and housing are presented in Chapter 3. Briefly, eight diets/treatments were offered *ad libitum* to broiler chickens, randomly designed into eight replicates per treatment. The treatments (Table 3.1) were maize and soya bean based diets supplemented with various commercial phytases.

4.2.1 Bone parameters

At 29 days of age one bird per pen (eight pens per treatment) was randomly selected from around the mean weight of the chickens in each pen. These birds were slaughtered according to standard commercial practice including electrical stunning (50-70 volts; 3-5 seconds), followed by exsanguination within 10 seconds of stunning. Both tibias were removed and frozen at -20 °C for further analysis. The left tibias were thawed, cleaned of adherent tissue and weighed. The tibias were kept cool until the breaking strength was determined using the three point destructive bending test prescribed by Fleming *et al.* (1998) using an Instron 3345 material testing machine (model 2519-107) fitted with a 3-point-bend rig with a load cell capacity of 5000 N and crosshead speed of 30 mm/min. The length of the tibia and mid diaphyseal diameter

was measured using a dial calliper with accuracy of 0.1mm. The centre point was marked on the diaphysis with ink and placed between two 14 mm retaining bars, set 38 mm apart. The 18 mm diameter crosshead probe approached the anterior side of the tibia at 30 mm/min until the bone was broken. The breaking strength (N) was recorded as the point of maximum load before failure occurred.

The right tibia was thawed, cleaned of adherent tissue and cartilage before the weight of the tibia was recorded. Dry matter of tibias was determined according to the Official Method 934.01 of the AOAC (2002). Tibias were placed in a dry porcelain cubicle and dried at 100 °C for 24 hours. Subsequently the tibia and cubicle was placed in the desiccator for 30 min to cool and then weighed. Tibias were defatted in petroleum ether for 48 hours (Rama Rao & Reddy, 2001) and were broken in half beforehand to facilitate fat extraction. Fat free dry bone weight was determined by drying the tibias at 100 °C for 24 hours. Subsequently, fat free bone ash percentage was determined after placing the tibia in a furnace for 24 hours at 600 °C (Zhang & Coon, 1997). All the weight measurements of the bone were determined using a Mettler AE 200 scale (Mettler-Toledo, Switzerland) with 0.0001g accuracy. Mineral analysis of the tibia was performed at the Institute of Animal Production, Western Cape Department of Agriculture. Mineral composition was determined according to the combustion method (method no. 6.1.1) in ALASA (1996). The tibia ash samples had 5 ml of 6 M hydrochloric acid added to each sample individually. The samples were placed in an oven for 30 minutes at 50 °C, after which 35 ml distilled water was added and the solution filtered into a bottle and made up to a final volume of 50 ml with distilled water. Elements were measured on an iCAP 6000 Series Inductive Coupled Plasma (ICP) Spectrophotometer (Thermo Electron Corporation, Strada Rivoltana, 20090 Rodana, Milan, Italy) fitted with a vertical quartz torch and Cetac ASX-520 autosampler. Element concentrations were calculated using iTEVA Analyst software.

4.2.2 Carcass characteristics

At 32 days of age, one bird per pen (eight pens per treatment) was selected with a body weight close to the mean weight of its pen and its live weight was recorded. These birds were slaughtered according to standard commercial practice, including electrical stunning followed by exsanguination. The broilers were scalded; defeathered and eviscerated (this included the removal of all the internal organs, feet and neck). Initial muscle pH (pH_i) of the breast and thigh was determined 15 minutes *post mortem* using a calibrated portable Crison pH25 meter (Alella, Barcelona) by means of a small incision in the centre of both the thigh and breast muscle. Ultimate muscle pH (pH_u) was determined 24 hours *post mortem* in the same manner and position as described for pH_i . Following the initial pH measurement, the carcasses were hung in cold storage at 4°C for 24 hours.

Live weight, hot carcass weight and chilled carcass weight 24 hours *post mortem* were recorded. Dressing percentage was calculated as the percentage difference between the live weight of the chicken and the weight of the hot carcass. Commercial portion yields were determined by first cutting the cold carcasses in half using a portion cutter. Subsequently, the thigh and drumstick were removed by cutting above the thigh towards the acetabulum and behind the pubic bone. The drumstick and thigh were separated by cutting perpendicular towards the joint connecting these two cuts. The wings were removed from the carcass by

cutting through the joint between the scapula and the coracoid. The separate portions were weighed using a Mettler PC 4400 scale (Mettler-Toledo, Switzerland). Percentage component yields were then calculated by expressing these weights as a percentage relative to chilled carcass weight. Subsequently the breast was dissected into muscle, skin and subcutaneous fat combined and bone. These fractions were weighed and expressed as a percentage relative to the total breast weight.

The skin of the thigh was removed; the dissected breast muscle together with the exposed thigh muscle was placed on a flat surface and allowed to bloom for 15 min (Warriss, 2000) at 8 °C. Meat colour (L^* , a^* , b^* measurements) were measured using a CIE-Lab colour meter (BYK-Gardner GmbH, Gerestried, Germany) where L^* represents lightness, a^* represents redness and b^* represents yellowness (Nollet *et al.*, 2007). Measurements were taken in triplicate over the total area of the muscle and the average calculated. According to Warriss (2000), a blooming period between 15 to 60 minutes is adequate. During the recording of CIE-Lab measurements the samples were randomly divided into two groups as a result of space limitations. Due to logistics, the blooming period for the two groups differed (15 minutes and 40 minutes). Statistical analyses were adjusted to account for the variation in time between the colour measurements of the two groups.

4.2.3 Statistical analysis

Statistical analysis for all the parameters except CIE-Lab measurements were analysed using the general linear models (GLM) and ANOVA procedures of SAS (2009) with treatment as the main effect. A two- way ANOVA was performed on the CIE-Lab measurements to declare the variation due to differences in blooming period between the two groups. All the parameters were tested for normality and homoscedasticity before analysis. Bonferroni post hoc test was applied for all mean comparisons (SAS, 2009). Differences were considered significant if P -values were less than 0.05.

4.3 Results and Discussion

4.3.1 Bone breaking strength

The results for bone breaking strength in (Table 4.1) are expressed as the total force in Newton (N) required to break the tibia or the breaking force per gram of bone required. Tibia breaking strength, expressed as N/g, was affected by dietary available P (aP) and Ca levels.

Table 4.1 Mean (\pm standard deviation) of tibia breaking strength of broilers slaughtered at 29 days of age receiving different dietary phytase treatments

Treatment	Tibia strength (N)	Tibia strength (N/g)
CON	241.46 ^c \pm 39.83	28.48 ^b \pm 5.89
NEG1000	143.22 ^a \pm 15.39	19.27 ^a \pm 1.26
NEG1500	153.06 ^{ab} \pm 18.56	19.15 ^a \pm 1.92
PHY	202.78 ^{abc} \pm 35.25	24.87 ^{ab} \pm 4.15
HP1000	218.66 ^{bc} \pm 39.19	26.26 ^{ab} \pm 5.55
NATU	221.70 ^c \pm 55.35	26.93 ^{ab} \pm 6.26
RZ	197.87 ^{abc} \pm 42.40	25.56 ^{ab} \pm 6.23
HP1500	185.85 ^{abc} \pm 30.38	23.56 ^{ab} \pm 3.70
P-value	< 0.001	0.007

^{a,b} Means within columns with different superscripts differ significantly ($P < 0.05$)

CON: Normal specification of full feed

NEG1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos

NEG1500: Normal specification of full feed minus matrix values of 1500 FYT HiPhos

PHY: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 500 FTU Phyzyme phytase

HP1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 1000 FYT HiPhos phytase

NATU: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 500 FTU Natuphos phytase

RZ: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 1500 FYT Ronozyme phytase

HP1500: Normal specification of full feed minus matrix values of 1500 FYT HiPhos + 1500 FYT HiPhos phytase

A significant increase for tibia breaking force (N/g) was observed in the CON treatment group compared to the NEG1000 and NEG1500 treatment groups, but all the phytase treatment groups were intermediate to the negative controls (NEG1000 and NEG1500) and the positive control (CON). However, when tibia breaking force was expressed in N, without consideration of bone weight, supplementing the NEG1000 diet with 500 FTU Natuphos or 1000 FYT HiPhos increased ($P < 0.05$) the breaking strength compared to the NEG1000 treatment group.

Similar to these findings, Shaw *et al.* (2011) reported that 1000 FYT HiPhos supplementation was able to increase tibia strength of 21 day old broilers compared to a negative control treatment group (npP = 0.22), although the increment was not big enough and the tibia breaking strength was still significantly less than the tibia strength of broilers receiving the positive control diet (3.8 g/kg npP, 9.4 g/kg Ca). Whereas Aureli *et al.*

(2011), who supplemented diets (Total P = 4.1 g/kg, Ca = 6.0 g/kg) with 1000 FYT HiPhos liquid, reported that the tibia breaking strength of 22 day old broilers receiving the phytase treatment were significantly higher than the positive (Total P = 5.6 g/kg, Ca = 6.0 g/kg) and negative (Total P = 4.1 g/kg, Ca = 6.0 g/kg) control treatment groups. In the present study, tibia breaking strength of chickens supplemented with Ronozyme and Phyzyme were intermediate to the NEG1000 and CON treatment groups. However, Shaw *et al.* (2010) showed that the supplementation of 750 FYT Ronozyme or 500 FTU Phyzyme to diets low in P (npP = 2.5 g/kg) can significantly increase tibia breaking strength so as to be similar to that of chickens raised on diets containing 4.5 g/kg npP. Onyango *et al.* (2004) speculated that different results in the literature regarding tibia breaking strength could be due to differences in age of the broilers, the crosshead speed of the Instron's probe, handling of the bones before testing and the site at which the shearing was done.

4.3.2 Bone ash and mineral content

Fat free dry bone weight of the NEG1000 treatment was significantly lower than the CON, HP1000 and NATU treatment groups (Table 4.2). Although there were no further differences for fat free dry bone weight, a tendency ($P = 0.063$) towards a difference was observed between the CON and NEG1500 treatment groups. Diets with inadequate amounts of aP often lead to low levels of bone mineralisation and therefore a reduction in tibia ash content (Santos *et al.*, 2008; Shaw *et al.*, 2011). Results from this study support such findings. Bone ash content (Table 4.2) of the CON treatment group was significantly higher than the NEG1000 and NEG1500 treatment groups. All the other treatment groups were intermediate to the negative and positive controls. In contrast with these finding, Rousseau *et al.* (2012) reported a significant increase in tibia ash when 500 FTU Natuphos was supplemented to diets with different Ca and npP concentrations. Moreover, Shaw *et al.* (2011) found that the supplementing HiPhos (1000 FYT) to broiler diets with 1.6 g/kg less npP than the positive control significantly increased the tibia ash content, but the increment was not sufficient and the tibia ash content of the treatment group was still significantly less than the positive control. The relative amounts and properties of the mineral content together with the organic matrix in the skeleton have an effect on bone strength (Boskey *et al.*, 1999). Furthermore, it is possible that variations in mineral composition (Ca:P ratio) can disrupt the structure of the hydroxyapatite crystal in the bone and may weaken the bone and have an effect on bone strength (Thorp & Waddington, 1997).

In the current study, different aP and Ca levels between the CON and NEG treatment groups and the supplementation of NEG groups with phytase had no effect on the Ca%, P% of the tibia (Table 4.2) Similarly, Han *et al.* (2009) reported no differences in Ca% in the tibia of broilers supplemented with phytase, but they did however report an increase in P content of the tibia as a result of phytase supplementation. No differences were observed for Ca:P ratio between the treatment groups. The Ca:P ratio in the tibia for all the treatment groups were slightly higher, but close to the expected value of 1.67:1. This value resembles the molar Ca:P ratio of the calcium phosphate (hydroxyapatite) occurring in the bone matrix (Pellegrino & Biltz, 1968). In the current trial, the improvements in bone weight, bone ash and tibia breaking force in treatment groups supplemented with phytase are indications of improved bone mineralisation which is due to increased

availability of P and Ca due to liberation of these minerals from phytate. However, the lack in significant differences for P and Ca content of the tibia was unexpected.

Table 4.2 Mean (\pm standard deviation) bone ash, tibia breaking strength and calcium and phosphorus content of the fat free tibia obtained from broilers fed different dietary phytase treatments

Treatment	Fat free dry bone weight (g)	Fat free bone ash (%)	Ca as % of bone ash	P as % of bone ash	Ca:P of bone ash
CON	3.30 ^b \pm 0.29	52.14 ^b \pm 2.12	33.09 \pm 3.49	18.44 \pm 0.66	1.79 \pm 0.13
NEG1000	2.77 ^a \pm 0.34	48.95 ^a \pm 1.38	32.50 \pm 3.90	17.92 \pm 0.78	1.81 \pm 0.16
NEG1500	2.90 ^{ab} \pm 0.31	48.98 ^a \pm 1.90	33.44 \pm 3.50	18.65 \pm 1.03	1.75 \pm 0.09
PHY	3.14 ^{ab} \pm 0.19	50.28 ^{ab} \pm 1.48	32.69 \pm 2.37	18.70 \pm 1.18	1.73 \pm 0.08
HP1000	3.22 ^b \pm 0.28	51.13 ^{ab} \pm 1.14	31.74 \pm 2.70	18.38 \pm 1.16	1.72 \pm 0.07
NATU	3.25 ^b \pm 0.19	50.31 ^{ab} \pm 2.09	31.98 \pm 2.53	18.59 \pm 0.98	1.70 \pm 0.10
RZ	3.05 ^{ab} \pm 0.19	50.30 ^{ab} \pm 1.16	31.19 \pm 3.86	18.30 \pm 1.31	1.79 \pm 0.10
HP1500	3.15 ^{ab} \pm 0.16	50.51 ^{ab} \pm 0.98	31.44 \pm 3.36	18.23 \pm 0.96	1.72 \pm 0.11
P- value	0.001	0.004	0.843	0.832	0.511

^{a,b} Means within columns with different superscripts differ significantly ($P < 0.05$)

CON: Normal specification of full feed

NEG1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos

NEG1500: Normal specification of full feed minus matrix values of 1500 FYT HiPhos

PHY: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 500 FTU Phyzyme phytase

HP1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 1000 FYT HiPhos phytase

NATU: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 500 FTU Natuphos phytase

RZ: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 1500 FYT Ronozyme phytase

HP1500: Normal specification of full feed minus matrix values of 1500 FYT HiPhos + 1500 FYT HiPhos phytase

4.3.3 Dressing percentage

Carcass dressing percentage is influenced by muscle growth and/or visceral growth. Dressing percentage decreases when abdominal fat, which is considered as a waste in broiler production, or visceral organ weight, increases (Salma *et al.*, 2007). No differences in dressing percentage were observed between treatments (Table 4.3). Similarly, Scheideler & Ferket (2000) did not observe differences in percentage carcass yield between broilers receiving diets with different npP levels or diets supplemented with 500 FTU Natuphos phytase. In contrast with these findings, Çimrin & Demirel (2008) reported higher dressing percentages in broilers receiving diets with adequate P and Ca levels (finisher: 4.4 g/kg aP, 8.5 g/kg Ca; grower: 4.4 g/kg aP, 8.5 g/kg Ca) compared to broilers receiving diets with lower Ca and P levels (finisher: 2.2 g/kg aP, 8.5 g/kg Ca; grower: 3.0 g/kg aP, 8.5 g/kg Ca). On the other hand, phytase supplementation (500 FYT Ronozyme) in the trial of Çimrin & Demirel (2008) did not significantly increase dressing percentage, yet supplementing phytase together with vitamin E and Selenium had an increasing effect on this parameter.

Table 4.3 Mean (\pm standard deviation) dressing percentage and percentage of muscle, skin and fat and bone of the breast obtained from broilers slaughtered at 32 days of age receiving different dietary phytase treatments

Treatment	Dressing percentage	% Muscle	% Skin and fat	% Bone
CON	67.41 \pm 1.71	81.17 \pm 5.10	5.87 ^a \pm 1.07	12.53 \pm 4.90
NEG1000	67.35 \pm 4.43	79.07 \pm 3.85	6.82 ^{ab} \pm 0.86	13.31 \pm 4.47
NEG01500	65.45 \pm 1.99	80.00 \pm 3.59	6.26 ^{ab} \pm 0.91	13.60 \pm 3.70
PHY	66.06 \pm 1.67	76.36 \pm 5.16	6.43 ^{ab} \pm 0.78	16.62 \pm 5.06
HP1000	66.17 \pm 3.09	79.16 \pm 5.21	7.93 ^b \pm 1.73	13.83 \pm 3.40
NATU	67.04 \pm 1.06	79.92 \pm 3.06	6.26 ^{ab} \pm 0.86	13.89 \pm 2.61
RZ	66.86 \pm 2.19	79.33 \pm 3.33	6.18 ^{ab} \pm 1.48	14.13 \pm 3.27
HP1500	67.21 \pm 2.19	81.59 \pm 4.32	6.13 ^{ab} \pm 1.49	12.21 \pm 4.19
P-value	0.705	0.373	0.039	0.529

^{a,b} Means within columns with different superscripts differ significantly ($P < 0.05$)

CON: Normal specification of full feed

NEG1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos

NEG1500: Normal specification of full feed minus matrix values of 1500 FYT HiPhos

PHY: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 500 FTU Phyzyme phytase

HP1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 1000 FYT HiPhos phytase

NATU: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 500 FTU Natuphos phytase

RZ: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 1500 FYT Ronozyme phytase

HP1500: Normal specification of full feed minus matrix values of 1500 FYT HiPhos + 1500 FYT HiPhos phytase

4.3.4 Carcass component yield

Phytase supplementation or nutrient composition (CON vs. NEG1000 or NEG1500) did not affect carcass component yield. These results are in agreement with Angel *et al.* (2006) who reported no differences in breast, wing, leg or barrel percentages between the positive (Starter npP content = 0.45; grower npP content = 0.32; finisher npP content = 0.33) and negative control group (Starter npP content = 0.25; grower npP content = 0.13 ; finisher npP content = 0.11) or between these groups and broilers receiving negative control diets supplemented with 600FYT Ronozyme phytase. In the same way, Scheideler & Ferket (2000) reported no differences in breast, wing and leg quarter weight of broiler supplemented with 500 FTU Natuphos phytase. On the other hand, Nagata *et al.* (2011) reported phytase to have an effect on breast yield and dressing percentage, but an interaction existed between phytase, energy and protein levels of the diets.

Table 4.4 Mean (\pm standard deviation) carcass component yield (%) of breast, thigh, leg, back and wing obtained from broilers slaughtered at 32 days of age receiving different dietary phytase treatments

Treatment	Breast %	Thigh %	Leg %	Back %	Wing %
CON	33.01 \pm 2.54	26.54 \pm 2.65	14.21 \pm 1.35	10.16 \pm 1.68	13.75 \pm 2.07
NEG1000	31.91 \pm 2.95	27.13 \pm 1.76	14.06 \pm 1.98	10.56 \pm 1.35	13.58 \pm 1.30
NEG1500	33.39 \pm 2.67	26.22 \pm 1.26	14.32 \pm 1.58	9.62 \pm 1.45	14.49 \pm 1.53
PHY	32.65 \pm 3.75	25.78 \pm 1.33	14.86 \pm 1.27	10.45 \pm 1.42	15.10 \pm 2.29
HP1000	31.47 \pm 1.08	27.53 \pm 1.03	13.94 \pm 1.46	11.40 \pm 1.20	13.72 \pm 1.27
NATU	34.22 \pm 2.71	26.93 \pm 1.15	13.99 \pm 0.73	10.73 \pm 1.6	13.94 \pm 1.96
RZ	32.38 \pm 2.96	26.88 \pm 1.19	15.67 \pm 0.72	11.25 \pm 1.13	13.57 \pm 1.82
HP1500	33.60 \pm 2.41	25.35 \pm 1.38	15.15 \pm 1.12	10.48 \pm 1.47	12.87 \pm 1.39
P-value	0.519	0.114	0.108	0.275	0.334

CON: Normal specification of full feed

NEG1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos

NEG1500: Normal specification of full feed minus matrix values of 1500 FYT HiPhos

PHY: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 500 FTU Phyzyme phytase

HP1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 1000 FYT HiPhos phytase

NATU: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 500 FTU Natuphos phytase

RZ: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 1500 FYT Ronozyme phytase

HP1500: Normal specification of full feed minus matrix values of 1500 FYT HiPhos + 1500 FYT HiPhos phytase

4.3.5 pH and CIE-Lab measurements

Phytase supplementation had no significant ($P > 0.05$) effect on breast or thigh pH values (Table 4.5). Treatments also had no significant effect on CIE L^* , a^* or b^* measurements for the breast muscle, but the L^* values of the thigh muscle was higher ($P < 0.05$) for the HiPhos treatment group compared to chickens in the RZ and NATU group (Table 4.6). Similarly, Han *et al.* (2009) found phytase supplementation had no effect on meat colour or muscle pH. Han *et al.* (2009) did however report lower pH_u and higher L^* measurements for all the treatment groups in the latter study compared to the average of all the treatments in the current study. Several studies have determined the pH and CIE-Lab measurements for normal colour poultry meat that is acceptable to the consumer (Fletcher, 1999; Van Laack *et al.*, 2000; Qiao *et al.*, 2001). A high correlation exists between muscle pH and meat colour (Mancini & Hunt, 2005). Dark meat is associated with a higher pH, whilst a lighter meat usually has a lower pH. In extreme cases, meat with a high pH can be characterised as being dark, firm and dry (DFD) and meat with a low pH can be pale soft and exudative (PSE) (Fletcher, 1999; Richardson & Mead, 1999). However, large variation exists between studies for the L^* measurement in “normal” colour meat. Fletcher (1999), Van Laack *et al.* (2000) and Qiao *et al.* (2001) reported L^* measurements of normal meat to be 45.6; 55.1 or 48 to 53, respectively. Furthermore, Van Laack *et al.* (2000) reported pH_u values of 5.96 for normal and 5.70 for pale coloured breast meat. In the current study, L^* and pH measurements indicated typically PSE meat. However, this phenomenon could not be linked to the specific dietary treatments (as this phenomenon occurred in all the treatments).

This phenomenon could be due to the fact that the meat samples used to measure meat colour in the current study were divided in two groups wherein the blooming period differed between the groups. Blooming period resulted in significant differences for L^* , a^* and b^* measurements in the breast and thigh (results not shown); the shorter blooming period increased the average L^* values. It is thought that the longer blooming period may have resulted in the surface of the muscle becoming more dry and thus less light (lower L^* values) being recorded. Environmental factors such as extreme ambient temperature, genetic factors, inadequate handling or insufficient stunning may play a role in inducing this phenomenon (Nollet *et al.*, 2007). However, to our knowledge, none of these factors affected the outcome of the study.

Table 4.5 Mean (\pm standard deviation) pH_i and pH_u of breast and thigh muscle obtained from broilers slaughtered at 32 days of age receiving different dietary phytase treatments

Treatment	pH_i Breast muscle	pH_u Breast muscle	pH_i Thigh muscle	pH_u Thigh muscle
CON	5.93 \pm 0.37	5.70 \pm 0.37	5.92 \pm 0.25	5.89 \pm 0.08
NEG1000	6.22 \pm 0.36	5.79 \pm 0.16	6.00 \pm 0.06	5.89 \pm 0.05
NEG01500	6.23 \pm 0.16	5.75 \pm 0.22	6.10 \pm 0.15	5.90 \pm 0.12
PHY	6.27 \pm 0.19	5.67 \pm 0.21	6.06 \pm 0.13	5.89 \pm 0.11
HP1000	6.17 \pm 0.21	5.67 \pm 0.27	6.00 \pm 0.18	5.83 \pm 0.08
NATU	6.04 \pm 0.22	5.74 \pm 0.33	5.85 \pm 0.10	5.84 \pm 0.09
RZ	6.21 \pm 0.22	5.77 \pm 0.26	6.01 \pm 0.06	5.86 \pm 0.09
HP1500	6.32 \pm 0.29	5.67 \pm 0.39	6.05 \pm 0.17	5.78 \pm 0.07
P-value	0.098	0.741	0.593	0.103

CON: Normal specification of full feed

NEG1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos

NEG1500: Normal specification of full feed minus matrix values of 1500 FYT HiPhos

PHY: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 500 FTU Phyzyme phytase

HP1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 1000 FYT HiPhos phytase

NATU: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 500 FTU Natuphos phytase

RZ: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 1500 FYT Ronozyme phytase

HP1500: Normal specification of full feed minus matrix values of 1500 FYT HiPhos + 1500 FYT HiPhos phytase

Table 4.6 Mean (\pm standard deviations) colour measurements (CIE-Lab) for breast and thigh muscle obtained from broilers slaughtered on 32 days of age receiving different dietary phytase treatments

Treatment	Breast		
	L*	a*	b*
CON	55.34 \pm 4.48	5.12 \pm 4.48	12.04 \pm 1.94
NEG1000	56.65 \pm 2.49	3.13 \pm 1.60	11.08 \pm 1.11
NEG1500	57.51 \pm 4.71	4.02 \pm 1.41	11.06 \pm 2.28
PHY	58.29 \pm 4.59	3.60 \pm 1.35	12.64 \pm 2.50
HP1000	57.51 \pm 2.68	3.91 \pm 1.55	11.75 \pm 1.66
NATU	55.34 \pm 4.03	3.78 \pm 1.20	11.64 \pm 1.29
RZ	56.98 \pm 3.30	3.03 \pm 0.90	11.44 \pm 1.69
HP1500	57.91 \pm 3.75	4.06 \pm 1.45	13.39 \pm 2.36
P value	0.220	0.114	0.284

Treatment	Thigh		
	L*	a*	b*
CON	55.60 ^{ab} \pm 2.30	4.94 \pm 1.15	11.21 \pm 2.02
NEG1000	56.39 ^{ab} \pm 4.72	4.29 \pm 0.93	11.49 \pm 2.21
NEG1500	56.80 ^{ab} \pm 4.22	4.59 \pm 0.79	11.31 \pm 1.03
PHY	57.06 ^{ab} \pm 3.05	4.53 \pm 1.79	11.60 \pm 1.64
HP1000	57.73 ^{ab} \pm 2.46	4.59 \pm 1.33	12.00 \pm 1.28
NATU	54.36 ^b \pm 2.26	5.13 \pm 1.02	12.52 \pm 0.75
RZ	55.30 ^b \pm 2.09	4.86 \pm 1.24	11.81 \pm 1.29
HP1500	59.55 ^a \pm 4.07	4.12 \pm 1.16	12.00 \pm 1.28
P value	0.022	0.813	0.837

^{a,b} Means within columns with different superscripts differ significantly ($P < 0.05$)

CON: Normal specification of full feed

NEG1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos

NEG1500: Normal specification of full feed minus matrix values of 1500 FYT HiPhos

PHY: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 500 FTU Phyzyme phytase

HP1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 1000 FYT HiPhos phytase

NATU: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 500 FTU Natuphos phytase

RZ: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 1500 FYT Ronozyme phytase

HP1500: Normal specification of full feed minus matrix values of 1500 FYT HiPhos + 1500 FYT HiPhos phytase

4.4 Conclusion

In the present study the negative control (NEG1000 and NEG1500) diets were formulated by subtracting the matrix values for 1000 FYT or 1500 FYT HiPhos from the positive controls (CON) diet. The difference in P and Ca was sufficient to create significant differences between positive and negative controls when tibia strength, fat free dry bone weight and fat free bone ash percentage were taken into account. Supplementing the CON1000 diet with 1000 FYT HiPhos was sufficient to significantly increase tibia breaking strength (N) and fat free dry bone weight so as to be similar to the positive control. Therefore the response to these two parameters confirmed the matrix values for HP1000. However, fat free bone ash percentage of the HP1000 group was intermediate to the CON and NEG1000 groups. Therefore although 1000 FYT HiPhos supplementation increased bone ash, it was in insufficient amounts to differ from the NEG1000 group to confirm the matrix value.

The same conclusion can be made for the 1500 FYT HiPhos. Supplementing the NEG1500 diet with 1500 FYT HiPhos increased tibia strength, fat free dry bone weight and fat free bone ash percentage so as to be similar to the CON treatment group, but increments were not large enough to result in significant differences between the HP1500 and NEG1500 groups. Therefore, when using these bone parameters as a response criteria, the conclusion can be made that HiPhos phytase supplementation did not liberate the amount of P and Ca indicated in the matrix values for the enzyme.

Furthermore, bone parameters did not significantly differ between broilers supplemented with different commercial phytases. Therefore all the commercial phytases were equally effective in liberating minerals that had an effect on bone mineralisation. Phytase supplementation did not influence the pH or colour measurements of the meat, the dressing percentage, carcass component yield or the percentage muscle and bone in the breast of broilers. The conclusion was made that phytase supplementation do not have any negative effects on carcass characteristics, therefore producers can supplement broiler diets with phytase without the risk of influencing meat quality.

4.5 Reference

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Chapter 5

The effect of commercial phytase enzymes on intestinal histomorphological measurements of broiler chickens

Abstract

Exogenous phytase enzymes show potential in improving the immune status and small intestinal morphology of broilers, but results in previous studies are inconsistent. In this study, the effects of commercial phytases (HiPhos, Ronozyme, Natuphos or Phyzyme) on organ weight, gizzard erosion score, gastrointestinal pH and histomorphological measurements of the different sections of the small intestine were investigated. Five thousand one hundred and twenty broiler chicks were randomly assigned to one of eight dietary treatments containing different levels and types of phytase. Treatments were: 1) Positive control (CON); 2) CON minus the matrix values for 1000 FYT HiPhos (NEG1000); 3) CON minus the matrix values for 1500 FYT HiPhos (NEG1500); 4) NEG1000 + 500 FTU Phyzyme (PHY); 5) NEG1000 + 1000 FTU HiPhos (HP1000); 6) NEG1000 + 500 FTU Natuphos (NATU); 7) NEG1000 + 1500 FYT Ronozyme (RZ); NEG1500 + 1500 FYT HiPhos (HP1500). Phytase supplementation did not influence the pH of the proventriculus, duodenum, jejunum or ileum, but cecal pH of chickens in the HP1500 was significantly higher than chickens in the NG1500 group. It was concluded that the lower pH in the cecum of chicks in the NEG1500 was due to increased substrate levels in the cecum, resulting in increased microbial fermentation. Treatment did not have an effect ($P > 0.05$) on absolute or relative organ weight, nor was mean villi height, villi area, crypt depth or villous height: crypt depth ratio in the duodenum affected ($P > 0.05$) by phytase supplementation or nutrient levels. Based on these organ weights and histomorphological measurements of the small intestine results, the conclusion was made that the increase in performance usually observed when broilers are supplemented with phytase are only due to its nutrient releasing capabilities and not due to improvements of the immune status or small intestinal morphology of broilers.

***keywords:** broilers, phytase, cecal pH, intestinal morphology, villi, crypt, gizzard, heart, liver spleen, cecum

5.1 Introduction

Poultry diets in South Africa are mainly plant based, consisting of maize and soya bean meal. Cereals and oilseeds contains Myo-inositol-1,2,3,4,5,6-hexakis dihydrogen phosphate, also known as phytic acid or phytate (Ravindran *et al.*, 1994; Selle & Ravindran, 2007). Phytic acid is an important component in plants and serves as the primary storage form of phosphorus (P) and inositol in seeds (Hídvégi & Lásztity, 2002), but approximately 85% and 62% of the phosphorus (P) in maize and soya beans is in the form of phytate

(Ravindran *et al.*, 1994). Phytic acid is generally considered as an anti nutritional factor in monogastric animal feeds as the phosphate groups in the acid are negatively charged and can thus form complexes with positively charged minerals (Davies & Olpin, 1979; Cheryan & Rackis, 1980; Lonnerdal *et al.*, 1989; Brink *et al.*, 1991), proteins (Hídvégi & Lásztity, 2002) and starch (Yoon *et al.*, 1983), rendering these nutrients unavailable for absorption.

So far phytase is the only enzyme known to hydrolyse phytate, subsequently releasing the phytate bound P and associated nutrients. Monogastric animals do not synthesise sufficient amounts of phytase to hydrolyse phytate. Therefore the development of exogenous phytase as a supplement in animal feeds was a very important discovery in animal nutrition (Cromwell, 2009).

For the current study it was hypothesised that phytase supplementation will release the phytate bound nutrients and therefore reduce the stress on the digestive tract, increase available nutrients and subsequently increase villous height and crypt depth. The surface of the small intestine contains finger-like projections known as villi, which increase the surface area of the small intestine and therefore increases its absorptive capacity (Silverthorn, 2007). At the base of the villi, tubular invaginations known as crypts extend down into the connective tissue (Silverthorn, 2007). Absorptive cells, known as enterocytes, originate from stem cells located within the crypt (Shen, 2009). A decrease in villi height has been noted in conjunction with a reduction in nutrient availability (Pluske *et al.*, 1996; Van Beers-Schreurs *et al.*, 1998). Nutrients within the lumen of the small intestine have also been shown to stimulate cell proliferation (Goodlad & Wright, 1984). What is more, a positive correlation exists between crypt depth and cell production within the crypt (Hedemann *et al.*, 2003). However, shorter villi and deeper crypts may decrease nutrient absorption and increase endogenous losses through increased loss of enterocytes, consequently decreasing the production performance of the animal (Xu *et al.*, 2003). Therefore the villous height: crypt depth ratio can possibly be used as an indicator of digestive capacity of the small intestine and a smaller ratio usually results in a decreased digestion and absorption capacity (Montagne *et al.*, 2003). According to Van der Klis & Jansman (2002) the desired villi: crypt ratio in the jejunum is 3.2:1.

Nutrient deficiencies impair the animal's immune status (Kwak *et al.*, 1999). For example, low arginine levels in a diet can lead to under development of organs, including the lymphoid organs (Kwak *et al.*, 1999). The bursa of Fabricius, thymus and spleen are examples of lymphoid organs and form part of the avian immune system (Yegani & Korver, 2008). The effective development of these organs is very important for optimal immune responses (Kwak *et al.*, 1999). Dietary factors such as Se, vitamin E (Huff *et al.*, 2004), aflatoxins (Hamilton *et al.*, 1972) and antibiotics has been shown to have an effect on lymphoid organ weights in poultry, indicating adjustment of the immune response to the desired level (Zyla *et al.*, 2000). The well known trace metals that play a role in immunity function are Zn, Se, Mn and Cu (Kidd, 2004). Phytase has the ability to increase the digestibility or retention of Zn (Brenes *et al.*, 2003), Mn, Cu and P (Cowieson *et al.*, 2006) and therefore, phytase supplementation might have an effect on the immune status of the animal. Ghahri *et al.* (2012) suggested that the gut wall could be directly irritated by phytate or phytate might increase intestinal microflora growth that may cause inflammation which in turn triggers an immune response and also damages the cell lining of the gastrointestinal tract. An inappropriate immune response will unnecessarily depress performance and increase production costs (Collett *et al.*, 2005). Pirgozliev *et al.* (2005) also

suggested that phytase has a positive effect on the health status of broilers when he noted decreased levels of sialic acid in the excreta of supplemented chickens. The sialic acid is related to mucin excretion due to bacterial infections, cellular senescence and pathological conditions. Therefore a reduction of sialic acid in excreta of phytase supplemented broilers might be an indication that phytase benefits the health of broilers (Pirgozliev *et al.*, 2005) and can therefore be one of the mechanisms that contributes to the mode of action of phytase.

This study was designed to characterise possible changes in the gastrointestinal tract and organ size of broilers in response to different commercial phytases and supplementation levels.

5.2 Materials and Methods

Details on the experimental outlay, animals, diet and housing are presented in Chapter 3. Briefly, eight diets were offered *ad libitum* to broiler chickens which were randomly designed to eight treatment groups (Table 3.1) with eight replicas per treatment. The treatments were maize and soya bean meal based diets supplemented with one of four commercial phytases. At 29 days of age, one bird per pen was randomly selected from around the mean weight of the chickens in each pen. These birds were slaughtered according to standard commercial practice including electrical stunning followed by exsanguinations.

5.2.1 Organ weights

The heart, liver, spleen and bursa of Fabricius were excised from the fresh carcass and weighed using a Mettler PC 4400 laboratory scale (Mettler-Toledo, Switzerland). The gizzard was removed and cut open longitudinally and rinsed under running water. Once rinsed it was scored for gizzard erosion on an ordinal scale as described in Table 5.1.

Table 5.1 Gizzard Erosion scoring description

Score	Description
0	No erosion
1	Light erosion (roughness of epithelia)
2	Modest erosion (roughness and gaps)
3	Severe erosion (roughness, gaps and ulcers on stomach wall showing slight haemorrhaging)
4	Extreme erosion (roughness, gaps and haemorrhagic ulcers on stomach wall and separation of epithelia from stomach wall)

5.2.2 pH measurements

After the removal of the organs, gut samples were taken of the duodenum (on the gizzard side of the duodenum at the start of the pancreas), jejunum (approximately in the centre) and the ileum (5mm from Meckel's diverticulum to the ileocecal junction) within 15 minutes *post mortem*. The pH of the duodenum,

jejunum, ileum, proventriculus and cecum were measured using a calibrated (standard buffers pH 4.0 and 7.0 at 25°C) portable Crison pH25 meter (Alella, Barcelona) by inserting the pH electrode into the centre of the area of the digestive tract to be measured. The probe was thoroughly rinsed with distilled water between readings.

5.2.3 Histomorphological samples

The duodenum and jejunum samples were rinsed with a 0.9% saline solution and fixed in a 10% buffered formalin solution. These samples were maintained in the formalin at room temperature for a period of 30 days and submitted to the Stellenbosch University School of Medicine histology lab (Tygerberg campus) for processing. Samples were processed according to the method described in Presnell & Schreiber (1997). The processing consisted of washing, trimming, dehydration with alcohol, clearing with xylene and impregnation with paraffin wax. Tissue sections of about 3 to 4 µm were cut by a microtome, fixed on slides and stained using haematoxylin and eosin (H&E). Slides were examined by using the 2.5X magnification objective lens of a Zeiss Axioskop2 light microscope, equipped with a digital camera. Images were analysed using Axiovision image-analysis software, version 4.7.2 (Carl Zeiss microscopy). Images were viewed to measure the villi height, villi area and crypt depth as demonstrated on Plate 1. Villi height and area were measured from the tip of the villi to the villous-crypt junction for 10 consecutive intact villi. Crypt depth was estimated by measuring 10 crypts per section. Crypt depth was defined as the vertical distance from the villous-crypt junction to the lower limit of the crypt.



Plate 1 Photomicrograph of cross section of the duodenum intestinal mucosa from a broiler receiving phytase supplementation (VH: indicating villous height, CD: indicating crypt depth and VA: indicating villous area)

5.2.4 Statistical analysis

Statistical analysis for all the parameters except gizzard erosion were analysed using the general linear models (GLM) procedure of SAS (2009). Parameters were tested for normality and homoscedasticity before analysis. Welch's variance-weighted ANOVA test was applied when the assumption for homoscedasticity was rejected. Means were separated with a Bonferroni post hoc test (SAS, 2009). Significance was declared at $P \leq 0.05$. Gizzard erosion scores were analysed using the Chi-squared test of SAS (2009).

5.3 Results and Discussion

5.3.1 Organ weight and gizzard erosion

The absolute weight of the organs and the weight of the organs as a percentage relative to the body weight are shown in Table 5.2 and Table 5.3 respectively. In the present study, commercial phytase supplementation to maize and soya bean diets had no effect ($P > 0.05$) on liver, gizzard or heart weights. These results are consistent with the report by Viveros *et al.* (2002) that the supplementation of phytase to broiler diets had no effect on relative or absolute organ weight.

Table 5.2 Mean organs weight (\pm standard deviation) obtained from broilers slaughtered at 29 days of age receiving different dietary phytase treatments

Treatment	Gizzard (g)	Heart (g)	Liver (g)	Spleen (g)	Bursa (g)
CON	27.3 \pm 8.7	9.3 \pm 2.2	41.3 \pm 3.9	1.9 \pm 0.4	3.5 \pm 0.9
NEG1000	26.4 \pm 7.7	9.1 \pm 1.1	40.4 \pm 3.4	1.5 \pm 0.4	3.3 \pm 1.0
NEG1500	31.0 \pm 6.5	9.1 \pm 1.5	41.3 \pm 4.6	1.5 \pm 0.2	3.8 \pm 1.0
PHY	31.6 \pm 5.5	10.1 \pm 1.6	38.8 \pm 6.2	1.4 \pm 0.3	3.4 \pm 1.5
HP1000	31.0 \pm 6.0	9.0 \pm 1.6	40.8 \pm 3.0	1.8 \pm 0.3	3.6 \pm 0.7
NATU	24.5 \pm 5.5	9.1 \pm 0.9	39.0 \pm 6.0	1.7 \pm 0.6	3.8 \pm 1.4
RZ	29.2 \pm 4.4	9.6 \pm 0.7	39.0 \pm 4.7	1.6 \pm 0.3	4.0 \pm 1.1
HP1500	28.8 \pm 4.4	9.2 \pm 1.6	38.2 \pm 3.4	1.8 \pm 0.4	3.5 \pm 1.3
P- value	0.269	0.780	0.754	0.167	0.908

CON: Normal specification of full feed

NEG1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos

NEG1500: Normal specification of full feed minus matrix values of 1500 FYT HiPhos

PHY: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 500 FTU Phyzyme phytase

HP1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 1000 FYT HiPhos phytase

NATU: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 500 FTU Natuphos phytase

RZ: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 1500 FYT Ronozyme phytase

HP1500: Normal specification of full feed minus matrix values of 1500 FYT HiPhos + 1500 FYT HiPhos phytase

Table 5.3 Mean organ weight relative to body weight (\pm standard deviation) obtained from broilers slaughtered at 29 days of age receiving different dietary phytase treatments

Treatment	Gizzard (%)	Heart (%)	Liver (%)
CON	1.73 \pm 0.59	0.59 \pm 0.13	2.61 \pm 0.24
NEG1000	1.70 \pm 0.49	0.59 \pm 0.07	2.61 \pm 0.24
NEG1500	2.01 \pm 0.44	0.58 \pm 0.10	2.66 \pm 0.23
PHY	2.00 \pm 0.35	0.64 \pm 0.10	2.46 \pm 0.38
HP1000	1.97 \pm 0.45	0.56 \pm 0.09	2.58 \pm 0.16
NATU	1.55 \pm 0.35	0.57 \pm 0.05	2.45 \pm 0.36
RZ	1.87 \pm 0.29	0.62 \pm 0.04	2.50 \pm 0.27
HP1500	1.81 \pm 0.29	0.58 \pm 0.11	2.40 \pm 0.22
P-value	0.285	0.727	0.480

CON: Normal specification of full feed

NEG1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos

NEG1500: Normal specification of full feed minus matrix values of 1500 FYT HiPhos

PHY: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 500 FTU Phyzyme phytase

HP1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 1000 FYT HiPhos phytase

NATU: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 500 FTU Natuphos phytase

RZ: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 1500 FYT Ronozyme phytase

HP1500: Normal specification of full feed minus matrix values of 1500 FYT HiPhos + 1500 FYT HiPhos phytase

An increase in the lymphoid organ weight can be considered as an indication of an improvement of the immune system (Nourmohammadi *et al.*, 2011), but it is important to keep in mind that an excessive or inappropriate immune response will unnecessarily depress performance (Collett *et al.*, 2005). In the current study, phytase supplementation or nutrient composition (no differences between CON, NEG1000 and NEG1500) had no effect ($P > 0.05$) on the weights of the spleen, bursa or spleen:bursa ratio (Table 5.4). These findings are in agreement with results reported by Smulikowska *et al.* (2010) and Nourmohammadi *et al.* (2011) who supplemented broiler diets with 1000 FTU Natuphos and also reported no significant effects.

In contrast, other studies indicated that phytase may have an effect on the immune system. Zyla *et al.* (2004) noted a decrease in bursa weight (adjusted for final body weight) when Ronozyme phytase was supplemented to maize soya bean diets. It was concluded that the degradation products from phytate that were not dephosphorylated completely might influence the chicken's immune system. Then again, in an earlier study, Zyla *et al.* (2000) reported a significant increase in bursa weights in 21 day old broilers supplemented with phytase together with acid phosphatase, pectinase and citric acid.

Thymus weight was not recorded in the current study, but Nourmohammadi *et al.* (2011) reported an increased thymus weight in broilers supplemented with 500 FTU Natuphos. The conclusion was made that phytase supplementation might have an impact on immune system response, increasing the number of lymphocytes in the thymus and thereby obtaining a higher disease resistance. However, due to the lack of response to any of the phytase supplementation on the lymphoid organ weights in the current trial and due to

the controversial results in literature, the effect that phytase supplementation has on the broiler immune system remains uncertain.

Table 5.4 Relative lymphoid organ weight percentage of body weight (\pm standard deviation) and ratio obtained from broilers slaughtered at 29 days of age receiving different dietary phytase treatments

Treatment	Spleen %	Bursa %	Spleen:Bursa
CON	0.12 \pm 0.03	0.22 \pm 0.06	0.60 \pm 0.33
NEG1000	0.09 \pm 0.03	0.21 \pm 0.06	0.47 \pm 0.17
NEG1500	0.10 \pm 0.01	0.25 \pm 0.06	0.43 \pm 0.16
PHY	0.09 \pm 0.02	0.21 \pm 0.09	0.49 \pm 0.20
HP1000	0.11 \pm 0.02	0.23 \pm 0.04	0.51 \pm 0.11
NATU	0.11 \pm 0.04	0.24 \pm 0.08	0.49 \pm 0.22
RZ	0.11 \pm 0.02	0.25 \pm 0.07	0.43 \pm 0.10
HP1500	0.12 \pm 0.02	0.22 \pm 0.08	0.63 \pm 0.33
P-value	0.291	0.881	0.529

CON: Normal specification of full feed

NEG1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos

NEG1500: Normal specification of full feed minus matrix values of 1500 FYT HiPhos

PHY: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 500 FTU Phyzyme phytase

HP1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 1000 FYT HiPhos phytase

NATU: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 500 FTU Natuphos phytase

RZ: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 1500 FYT Ronozyme phytase

HP1500: Normal specification of full feed minus matrix values of 1500 FYT HiPhos + 1500 FYT HiPhos phytase

5.3.2 Gizzard erosion

A number of factors that provoke gizzard erosion have been identified. These factors include mycotoxins (Hoerr *et al.*, 1982; Dorner *et al.*, 1983), dietary copper sulphate levels (Fisher *et al.*, 1973), stress (Džaja *et al.*, 1996), histamine and histamine agonists such as gizzerosine (Džaja *et al.*, 1996). Gizzerosine is formed by the reaction of histamine or histidine during overheating of fishmeal (Tao *et al.*, 2012). Incidences of severe gizzard erosion were low during the current trial (Table 5.5). Therefore the conclusion can be made that none of the diets contained mycotoxins or histamine agonists which may negatively affect production.

Table 5.5 Number of observations per category of gizzard erosion scores recorded from broilers receiving different dietary phytase treatments

Treatment	Gizzard score				
	0	1	2	3	4
CON	0	2	3	1	2
NEG1000	2	2	2	0	2
NEG1500	3	5	0	0	0
PHY	5	2	1	0	1
HP1000	1	3	1	2	1
NATU	2	3	0	2	1
RZ	1	4	2	1	0
HP1500	2	4	2	0	0
Chi-Square P value	0.334				

CON: Normal specification of full feed

NEG1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos

NEG1500: Normal specification of full feed minus matrix values of 1500 FYT HiPhos

PHY: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 500 FTU Phyzyme phytase

HP1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 1000 FYT HiPhos phytase

NATU: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 500 FTU Natuphos phytase

RZ: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 1500 FYT Ronozyme phytase

HP1500: Normal specification of full feed minus matrix values of 1500 FYT HiPhos + 1500 FYT HiPhos phytase

5.3.3 The pH of the digestive tract

The digestive/absorptive surface of the small intestine is exposed to luminal fluid. The luminal fluid determines the pH, ionic and osmolarity of the micro environment surrounding the brush border membrane and its active enterocytes (Mitchell *et al.*, 2008). The health of the chicken, the kind of nutrients in the digesta as well as the gut microflora, all influence the intestinal pH (Rahmani *et al.*, 2005). The pH in specific areas of the gastrointestinal tract affects nutrient digestion and absorption (Rahmani *et al.*, 2005).

The pH of various areas in the digestive tract is presented in Table 5.6. Current study data indicate that phytase supplementation had no effect ($P > 0.05$) on pH values in the duodenum, jejunum or ileum of the broilers, which agrees with the findings of Nourmohammadi *et al.* (2011). Chickens in the NEG1500 treatment group had significantly lower cecal pH values compared to chickens in the HP1500 group. It was speculated that most phytate bound nutrients were not digested and absorbed in the small intestine of chickens in the NEG1500 group. Therefore greater amounts of nutrients flowed through to the cecum. An increase of nutrients in the cecum results in increased substrate levels for microbial fermentation, which in turn increases volatile fatty acid production and decreases cecal pH (Rayssiguier & Remesy, 1977). The conclusion was made that 1500 FYT HiPhos released phytate bound nutrients, rendering it available for absorption in the small intestine. Therefore lower levels of substrate were available for microbial

fermentation, resulting in a significant higher cecal pH compared to the cecal pH of chickens in the NEG1500 group. The crude fibre levels differed between the CON, NEG1000 and NEG1500 which may lead to differences in cecal pH, however 1500 FYT HiPhos was supplemented to the NEG1500 diet to formulate the HP1500 treatment. The HP1500 and NEG1500 treatments contained the same amount of fibre and fibre can thus be eliminated as a reason for the difference in cecal pH.

The luminal pH of the chickens in all the treatments increased from the proximal to the distal area of the small intestine as expected. The luminal pH values for the different parts of the small intestine for all the treatments in the current study revealed a marked acidity. These values differ from the generally accepted pH of the small intestine ranging between 6.5 to 7.5 (Simon & Igbasan, 2002), but resembles pH values reported by Engberg *et al.* (2002) for pellet-fed broilers. A reduction in intestinal pH is desirable (Engberg *et al.*, 2002) and may improve nutrient absorption (Rahmani *et al.*, 2005). As discussed in section 2.4.1.1, most of the commercial enzymes used in the present study have relatively good phytase activity at pH 5 and therefore the acidic conditions in the small intestine of the broilers in the current trial are beneficial for microbial phytase activity. For example, Natuphos shows activity between pH 2.5 to 6 with the optimal activity at pH 5.5. The average pH in duodenum and jejunum of broilers supplemented with Natuphos (NATU) was 5.08 and 5.77, respectively. Therefore, if the Natuphos phytase was resistant to proteolytic enzymes in the stomach of the broilers, the enzyme should have activity in the duodenum and jejunum. However, phytase activity rarely occur post duodenal (Yu *et al.*, 2004).

Table 5.6 Mean pH (\pm standard deviations) of various areas of the digestive tract obtained from broilers slaughtered at 29 days of age receiving different dietary phytase treatments

Treatment	Pro-ventriculus	Duodenum	Jejunum	Ileum	Cecum
CON	3.58 \pm 0.87	5.55 \pm 0.21	5.90 \pm 0.22	6.41 \pm 0.81	6.53 ^{ab} \pm 0.54
NEG1000	3.05 \pm 0.48	5.20 \pm 0.55	5.48 \pm 0.52	6.21 \pm 0.88	6.06 ^{ab} \pm 0.49
NEG1500	2.46 \pm 0.87	5.45 \pm 0.42	5.83 \pm 0.19	6.47 \pm 0.57	5.84 ^a \pm 0.38
PHY	2.80 \pm 0.60	5.41 \pm 0.38	5.88 \pm 0.46	6.71 \pm 0.61	6.40 ^{ab} \pm 0.61
HP1000	3.10 \pm 1.12	5.57 \pm 0.38	5.75 \pm 0.28	6.79 \pm 0.54	6.61 ^{ab} \pm 0.49
NATU	3.25 \pm 0.95	5.08 \pm 0.72	5.77 \pm 0.32	6.87 \pm 0.40	6.37 ^{ab} \pm 0.39
RZ	2.61 \pm 1.07	5.42 \pm 0.69	5.88 \pm 0.19	6.73 \pm 0.63	6.33 ^{ab} \pm 0.56
HP1500	2.29 \pm 0.57	5.64 \pm 0.26	5.85 \pm 0.09	6.63 \pm 0.66	6.77 ^b \pm 0.23
P value	0.061	0.294	0.180	0.492	0.007

CON: Normal specification of full feed

NEG1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos

NEG1500: Normal specification of full feed minus matrix values of 1500 FYT HiPhos

PHY: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 500 FTU Phyzyme phytase

HP1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 1000 FYT HiPhos phytase

NATU: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 500 FTU Natuphos phytase

RZ: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 1500 FYT Ronozyme phytase

HP1500: Normal specification of full feed minus matrix values of 1500 FYT HiPhos + 1500 FYT HiPhos phytase

Further, Nakano *et al.* (2001) concluded that phytase enzymes occur on the intestinal brush border membrane of rats and are activated at acidic pH. Similarly, Maenz & Classen (1998) proved brush border intestinal phytases to have optimum activity at pH 5.5 to 6.5. Therefore, the low intestinal pH in the small intestine of broilers in the current trial could be beneficial for intestinal and microbial phytase activity. As discussed in Chapter 2, high dietary Ca levels decreases phytate digestibility and by decreasing Ca levels, phytate digestibility can be increased (Mohammed *et al.*, 1991). It can be speculated that the low intestinal pH together with lower Ca levels in the Negative control diets contributed to phytate digestibility by means of intestinal phytase activity. This might be one of the reasons why the difference in live weight gain (Table 3.7, Chapter 3) and bone mineralisation (Table 4.2) between chickens in the negative controls and positive control were not as pronounced as expected.

5.3.4 Histomorphological Measurements

Due to the nutrient releasing effect of phytase, it is hypothesised that villi height and area will increase as a result of increased nutrient availability. This was however not the result in the current study. Treatments had no significant effect on villus height, villus area, crypt depth or villus height: crypt depth ratio in the duodenum (Table 5.7). In the jejunum, opposite results as expected were obtained. Villus height and villus height: crypt depth ratio of broilers in the NEG1500 treatment group were greater ($P < 0.05$) than the HP1000, HP1500 and NEG1000 treatment groups (Table 5.8).

Table 5.7 Mean (\pm standard deviation) villi height, villi area crypt depth and villus height: crypt depth ratio of the duodenum of broilers slaughtered on day 29 receiving different dietary phytase treatments

Treatment	Villi height (μm)	Villi area (μm^2)	Crypt depth (μm)	Villi height: crypt depth
CON	1394 \pm 307	187499 \pm 60017	215 \pm 22	6.60 \pm 1.82
NEG1000	1443 \pm 232	186734 \pm 45167	204 \pm 38	7.16 \pm 1.17
NEG1500	1715 \pm 297	195409 \pm 38391	202 \pm 33	8.58 \pm 1.30
PHY	1292 \pm 203	171442 \pm 48793	191 \pm 49	7.13 \pm 1.59
HP1000	1500 \pm 229	201056 \pm 25807	179 \pm 27	8.16 \pm 1.08
NATU	1469 \pm 228	217411 \pm 60101	195 \pm 36	7.89 \pm 2.63
RZ	1539 \pm 251	232011 \pm 42870	200 \pm 19	7.82 \pm 1.87
HP1500	1433 \pm 227	232551 \pm 55543	209 \pm 54	7.09 \pm 1.46
P value	0.108	0.211	0.735	0.375

CON: Normal specification of full feed

NEG1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos

NEG1500: Normal specification of full feed minus matrix values of 1500 FYT HiPhos

PHY: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 500 FTU Phyzyme phytase

HP1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 1000 FYT HiPhos phytase

NATU: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 500 FTU Natuphos phytase

RZ: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 1500 FYT Ronozyme phytase

HP1500: Normal specification of full feed minus matrix values of 1500 FYT HiPhos + 1500 FYT HiPhos phytase

Table 5.8 Mean (\pm standard deviation) villi height, villi area, crypt depth and villus height: crypt depth ratio of the jejunum of broilers slaughtered on day 29 receiving different dietary phytase treatments

Treatment	Villi height (μm)	Villi area (μm^2)	Crypt depth (μm)	Villi height: crypt depth
CON	1342 ^{ab} \pm 332	169051 \pm 49634	234 \pm 26	5.69 ^{ab} \pm 1.05
NEG1000	1222 ^a \pm 115	174180 \pm 30446	222 \pm 33	5.62 ^{ab} \pm 1.01
NEG01500	1578 ^b \pm 207	183972 \pm 28051	230 \pm 68	7.45 ^b \pm 2.74
PHY	1438 ^{ab} \pm 179	166548 \pm 36358	224 \pm 40	6.61 ^{ab} \pm 1.38
HP1000	1176 ^a \pm 163	158228 \pm 35368	254 \pm 81	4.86 ^a \pm 1.02
NATU	1436 ^{ab} \pm 148	177421 \pm 46715	264 \pm 41	5.52 ^{ab} \pm 0.71
RZ	1358 ^{ab} \pm 152	192612 \pm 53934	236 \pm 57	6.10 ^{ab} \pm 1.71
HP1500	1217 ^a \pm 263	168187 \pm 66678	265 \pm 71	4.72 ^a \pm 0.96
P-value	0.006	0.877	0.648	0.014

CON: Normal specification of full feed

NEG1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos

NEG1500: Normal specification of full feed minus matrix values of 1500 FYT HiPhos

PHY: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 500 FTU Phyzyme phytase

HP1000: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 1000 FYT HiPhos phytase

NATU: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 500 FTU Natuphos phytase

RZ: Normal specification of full feed minus matrix values of 1000 FYT HiPhos + 1500 FYT Ronozyme phytase

HP1500: Normal specification of full feed minus matrix values of 1500 FYT HiPhos + 1500 FYT HiPhos phytase

To the best of our knowledge, results on the effect of phytase on small intestinal morphology are scarce and conflicting results have been reported by authors. In the study of Smulikowska *et al.* (2010), shorter jejunum villus heights and deeper crypt depth were observed in chickens fed wheat, soya bean and rapeseed based diets low in non phytate phosphorus (npP) compared to diets with normal P levels. Furthermore, Khodambashi-Emami *et al.* (2013) reported an increase in villi height and villi height: crypt depth ratio in the duodenum and jejunum together with a decreased jejunum crypt depth when maize and soya bean based diets low in available phosphorus (aP) was supplemented with phytase. However, in the studies reported by Nourmohammadi & Afzali (2013), phytase supplementation increased crypt depth and decreased villus height: crypt depth ratio. In the current study, jejunum villi height of chickens in the NEG1500 significantly differed from the NEG1000 group whilst the CON treatment group was intermediary to these treatment groups. Therefore nutrient and P levels in the diets did not attribute to the differences observed. Development of the intestinal morphology may be a reflection of the health status of the gastrointestinal tract of chickens (Gao *et al.*, 2008). As mentioned in Chapter 3, mortality rates in the current study were high due to *Escherichia coli* infection of the chickens. Pathogens and their toxins may cause inflammation in the small intestine, resulting in increased villus atrophy. The possibility exists that the eight randomly selected chickens in the NEG1500 had a lower pathogen load than chickens in other treatments, contributing to the significant differences. The high standard deviations for jejunum villi heights and area in the CON and HP1500 treatments may be an indication that external factors influenced these parameters.

5.4 Conclusion

The commercial phytases used in the current study did not alter the weights of the gizzard, heart or liver, nor did phytase supplementation alter the weight of the spleen or bursa of Fabricius. Therefore the conclusion was made that phytase did not have an effect on the immune system as suggested in literature. No differences were observed between treatments for the pH of the proventriculus, duodenum, jejunum or ileum. The cecal pH of chickens in the NEG1500 treatment group was significantly lower than the HP1500 treatment group and it can therefore be concluded that higher amounts of unabsorbed nutrients were present in the cecum of NEG1500 chickens compared to HP1500 chickens. Chickens in the NEG1500 treatment group had significantly higher jejunum villi height and villi height:crypt depth ratio than chickens in the NEG1000, HP1000 and HP1500 treatment groups. The CON treatment group was intermediate to the two negative control groups. Therefore the conclusion can be made that nutrient composition (CON vs NEG1000 or NEG1500) or phytase supplementation were not the reason for the observed differences in villus height, but other confounding factors were responsible for the differences.

5.5 References

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Chapter 6

General conclusion

The primary objective of this study was to confirm the matrix values of a newly developed phytase enzyme (HiPhos) at two different inclusion levels. The following parameter measurements were used as response criteria to subsequently confirm the matrix values: live weight gain, tibia weight, tibia strength, fat free tibia ash percentage and mineral content of the tibia. The matrix value for 1000 FYT HiPhos was confirmed through the results obtained for tibia weight and tibia strength, but results for bone ash percentage was insufficient in confirming the matrix values. Reducing aP, Ca and nutrient levels in diets according to the matrix value of 1000 FYT HiPhos did not significantly influence live weight gain or mineral content of the tibia, therefore these parameters could not be used to confirm the matrix values. The matrix value of 1500 FYT HiPhos was confirmed by using live weight gain as response criteria. However, supplementing diets with 1500 FYT HiPhos increased bone ash percentage and tibia strength so as to be similar to the positive control, but increments were not high enough to create a significant difference between the HP1500 group and the negative control group, therefore the matrix values could not be confirmed when using bone parameters as a response criteria.

When evaluating the effect of the different phytase treatments on the production performance of the broilers it was seen that total feed and water intake were not influenced by phytase supplementation. Live weight, feed intake, FCR, EPEF, ADG, tibia strength, fat free tibia weight, fat free tibia ash and mineral content in the tibia did not differ between phytase treatments when supplemented at levels at which the phytases should have similar P equivalence. Therefore all the commercial phytases were equally effective. However, even though the cumulative live weight gain, ADG and EPEF of broilers in the HP1500 treatment group did not differ from other phytase treatments, it was the only treatment that showed significant differences for these three parameters compared to the Neg1500 treatment group. Therefore, when supplemented at higher levels, HiPhos shows potential for increasing the performance of broilers compared to broilers supplemented with the commercial phytases at the levels recommended by the manufacturers. Furthermore, supplementing diets with 1500 FYT HiPhos was the only phytase that increased cecal pH compared to the negative control group, it was concluded that smaller amounts of unabsorbed nutrients were present in the cecum of broilers in the HP1500 treatment group.

As pertaining to the broiler carcass characteristics, phytase supplementation did not influence dressing percentage, organ weight, carcass component yield, colour of the breast muscle or pH of the breast and thigh muscles. As pertaining to the intestinal histomorphological measurements of broiler chickens, phytase supplementation treatments did not influence the villi height or crypt depth in the duodenum. Differences for in villi heights and villi height: crypt depth in the jejunum were observed between treatments, but not as a result of nutrient composition or phytase supplementation.

In summary, the matrix values for 1000 FYT HiPhos and 1500 FYT HiPhos could not be confirmed nor disproved, nevertheless results from the current trial proved diets supplemented with HiPhos to be more economically viable when compared to the standard broiler diets. No major differences for the production and bone parameters were observed between the specific phytases evaluated, therefore the costs of these phytases can be the determining factor when nutritionists decide which commercial phytase to use. Furthermore, phytase supplementation has shown to have no effect on water intake, therefore farmers can supplement broiler diets with phytase without the concern of generating wet litter in the broiler house. Phytase supplementation did not influence carcass characteristics, therefore producers can supplement broiler diets with phytase without the risk of influencing meat quality. In addition, results of this study indicates that the increase in performance usually observed when broilers are supplemented with phytase is only due to its nutrient releasing capabilities of phytase and not due to improvements of the immune status or small intestinal morphology of broilers.